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STUDIES OF THE CARCINOGENIC ACTION IN THE RAT OF 2-NITRO-, 2-AMINO-, 2-ACETYLAMINO-, AND 2-DIACETYLAMINOFLUORENE AFTER INGESTION AND AFTER PAINTING [1] [2]

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INTRODUCTION

One group of carcinogens is active at or near the site of application, another group acts at distant but usually specific sites, while a third group produces distant tumors at a variety of sites. 2-acetylaminofluorene, (AAF), is one of the best examples of the third group (*1–11*). The eliciting of malignant tumors in many organs and tissues by the same chemical compound is a useful method of providing material for studying chemical and histo-chemical changes occurring in a variety of tissues during carcinogenesis. The synthesis in this laboratory of a large quantity of AAF provided 2-nitrofluorene (NF), reduced NF or 2-aminofluorene (AF), and 2-diacetylaminofluorene (diAAF), with which an exploratory study was made to determine (1) the influence of route of administration on carcinogenicity, and (2) the influence of reduction and acetylation on the nitrogen in the 2-position of the fluorene molecule by both methods of administration as indicated by the number and distribution of tumors as a guide for more detailed experiments now in progress. Subsequent reports will enlarge upon certain aspects of this paper.

Since these studies were started, a number of investigators have published results comparing the number and types of tumors induced by AAF and 2-aminofluorene (AF). Bielschowsky (*2*) in 1944 reported liver tumors in all of five male Wistar strain rats painted thrice weekly for 210 days with a 4 percent solution of 2-AF in acetone. He observed no alteration of the skin in the painted area. Harris (*12*) reported no difference in tumor incidence or types of tumors developing in rats of the Wistar strain

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[2] Presented in part at the annual meeting of the Am. Assoc. Cancer Research, Chicago, 1946.

after ingesting 2-AAF when compared with the tumors induced in rats by painting a 4 percent solution of 2-AF in benzene 2 or 3 times weekly for 62 applications on the abdominal skin of rats of the same strain. Wilson, DeEds, and Cox (*13*) observed tumors in female rats, ingesting for 403 days diets containing 0.016 and 0.031 percent AF, that were indistinguishable from tumors induced by AAF. Two animals receiving AF developed squamous-cell carcinoma of the head. Crystalline AF implanted subcutaneously did not induce tumors in rats, but several different types of tumors including leukemia were found in rats injected with a solution of AF in propylene glycol. The authors seem uncertain about the significance of these latter tumor observations because they were not types of tumors usually found in rats treated with AAF or AF. Animals injected with propylene glycol alone also developed leukemia. Kirby (*14*) observed distant tumors in three strains of mice after twice weekly paintings with a 4 percent solution of 2-AF in acetone. Ulceration, hyperkeratosis, and one squamous-cell carcinoma in strain dba were noted at the site of application of the carcinogen. There can be no doubt from the above experiments that AF is not only carcinogenic by ingestion, or after topical application, but also produces distant tumors at several sites.

MATERIALS AND METHODS

The synthetic basal diet (No. 143) was composed of the following:

	Gm.		Gm.
Vitamin-free casein	120.000	Vitamin mixture:	
Cerelose	744.000	Thiamine hydrochloride	.003
Salt mixture (185a) [1]	30.000	Riboflavin	.002
Coconut oil fortified with vitamins A and E [2]	2.500	Pyridoxine hydrochloride	.002
Glycerol	100.000	Calcium pantothenate	.014
Linoleic acid	.500	Cerelose	3.000
			1,000.021

[1] Morris and Robertson (*15*).

[2] Twenty-five grams fortified oil contained 2,000 units vitamin A ester* and 800 mg. α-tocopherol acetate.[1] Each constituent of the vitamin mixture was weighed individually and then thoroughly mixed with 30 gm. of cerelose before incorporating into 10 kg. of the diet.

* The vitamin A ester was generously donated for these experiments by Distillation Products Co., Inc., Rochester, N. Y.

The diet constituents, after being weighed into a mixing bowl, were thoroughly mixed by a mechanical dough mixer and stored in a cold room at +5° C. The control rats received basal diet 143. This was modified by the addition of the several compounds tested as follows:

diet 146 contained 0.05 percent or 2.37 millimoles of 2-nitrofluorene per kg., diet 147 contained 0.05 percent or 2.76 millimoles of 2-aminofluorene per kg., 148 contained 0.05 percent or 2.24 millimoles of 2-acetylaminofluorene per kg., and 149 contained 0.05 percent or 1.89 millimoles of 2-diacetylaminofluorene per kg.

Five grams of each compound dissolved in sufficient acetone to effect complete solution, were thoroughly mixed with 1 kg. of cerelose (corn

dextrose) in a large porcelain evaporating dish and allowed to stand at room temperature until the solvent was completely evaporated. During evaporation the mixture was stirred frequently to promote removal of the solvent. Sufficient amounts of the sugar containing the carcinogen were then incorporated into the diet mixture to produce a completed diet having the concentration of carcinogen indicated above.

All animals in both the ingestion and painting experiments were caged individually in wire-cloth-bottom cages. The carcinogen-containing diets were fed for 23 weeks, at which time the surviving animals were placed on the basal control diet until they developed gross tumors or when death appeared imminent. The animals were weighed weekly, and their food intake was also measured weekly during the entire experiment.

A 2 percent acetone solution of the carcinogens in the painting experiments was applied dropwise by means of a medicine type dropper to the clipped scapular region throughout the experiment. The rats were not able to ingest the carcinogens by licking when applied at this location. Three drops were applied three times weekly during the first 6 months, and thereafter six drops three times weekly. The dropper was made of a small rubber bulb attached to the upper end of the barrel of a 1 ml. tuberculin type syringe and a 3½ inch, 16 gauge hypodermic needle was attached to the opposite end. The same quantity of acetone was administered to control animals in the same way as to the treated animals. Separate droppers were used for each group.

All painted rats were fed throughout the experiment a diet of natural foodstuffs prepared in the form of pellets (*16*). These experiments were not intended to give information on the possible influence of diet on the carcinogenicity of these compounds. The diets were ones available in the laboratory at the time, and, to the extent that as yet unknown dietary constituents alter the carcinogenicity of the compounds studied, the interpretation of the results is difficult. Most (77) of the animals used were of a strain of highly inbred albino rats, the original stock having been procured from Dr. Cornelia Kennedy, Division of Agricultural Biochemistry, University of Minnesota, and have been designated the Minnesota strain. Because insufficient numbers of this strain were available to start all the experiments, one group of 20 was made up entirely of Buffalo strain rats. A few animals died early in the experiment in some groups and were replaced with 8 Wistar strain rats (*17*). All rats used were bred at the Institute. The variable strain susceptibility to the action of AAF was not known when these experiments were started in 1944. The sex of the rats was approximately equally distributed in the various groups. All tumors have been verified microscopically.

Preparation of compounds: The four compounds used in this study were prepared by first nitrating fluorene according to the method of Diels (*18, 19*). The 2-nitroderivative was reduced with zinc in the presence of $CaCl_2$ and then the acetylation of the 2-aminofluorene was carried out

by boiling with glacial acetic acid (*20*). The melting points of the resulting compounds were as follows:

2-nitrofluorene	m. p.	157.0° C	(uncorrected)
2-aminofluorene	m. p.	127.5° C	(uncorrected)
2-acetylaminofluorene	m. p.	194.0° C	(uncorrected)

A detailed description of the preparation of the 2-diacetylaminofluorene, only, is presented, since its preparation has not been previously recorded.

Preparation of 2-Diacetylaminofluorene from 2-Aminofluorene: 200 ml. acetic anhydride were heated to boiling while stirring in a 3-necked flask, 63 grams of 2-aminofluorene were gradually added during 40 minutes, after which the stirring and boiling were continued for 4 hours longer. The solution was then poured into an excessive amount of water. After standing over night the precipitate was filtered off and recrystallized from 1 liter of hot 95 percent alcohol, using norit. Fifty-six grams were obtained after two more recrystallizations from hot 95 percent alcohol. The compound showed a melting point of 134.1° C (corrected).

Analysis by Dr. A. T. Ness:

	Calculated	*Found*
C	76. 96	77. 07
H	5. 70	5. 82
Acetyl	32. 48	32. 86

Formula $C_{17}H_{15}NO_2$

Mol. wt. 265.30

Mixed m. p. of 2-AAF+2-diAAF

The mixture began to sinter at 121° and melted over the range of 143–162° uncorrected.[3]

EXPERIMENTAL RESULTS

The incidence of tumors as observed in this laboratory in rats given AAF usually appears to be directly related to length of survival; therefore, the length of survival of the animals in the several groups was compared. It was found that non-tumor-bearing rats receiving AF either orally or topically survived a somewhat shorter period than the AF-treated rats that developed tumors. This is shown by the data in table 1, where the average survival of AF-painted non-tumor-bearing rats was 310 days compared to 508 days for tumor-bearing rats, and the average survival of non-tumor-bearing rats fed AF was only 148 days, compared with survival of 284 days for tumor-bearing rats. The average survival of non-tumor-bearing rats of 646 days when painted with diAAF was somewhat longer than the 536 days for diAAF tumor-bearing rats, table 1. Differences in average survival in the other treated groups appears insignificant. The average length of survival in all groups of rats ingesting the stock diet was about twice that of animals receiving the synthetic diet.

[3] Personal communication from Dr. Elizabeth K. Weisburger.

TABLE 1.—*A summary giving for each compound and each route of administration the number, sex, and strain of rat used, the length of survival, and the quanity administered*

	Fluorene derivative								Control	
	2-nitro		2-amino		2-acetylamino		2-diacetylamino			
	Painting	Oral	Painting	Oral	Painting	Oral	Painting	Oral	Painting	Oral
No., sex, and strain of rat	1–W 9–M 7♂–3♀	9–M 6♂–3♀	10–M 1–W 6♂–5♀	11–M 5♂–6♀	7–M 2–W 4♂–5♀	20–B 10♂–10♀	9–M 2–W 5♂–6♀	M–12 5♂–7♀	M–4 W–2 3♂–3♀	M–6 3♂–3♀
Avg. survival (days):										
Tumor-bearing rats	554	308	508	284	495	241	536	58		
Non-tumor-bearing rats	560	310	310	148	497	200	646		617	280
Avg. estimated amount of carcinogen per rat-mg	69	756	53	537	58	580	65	216		

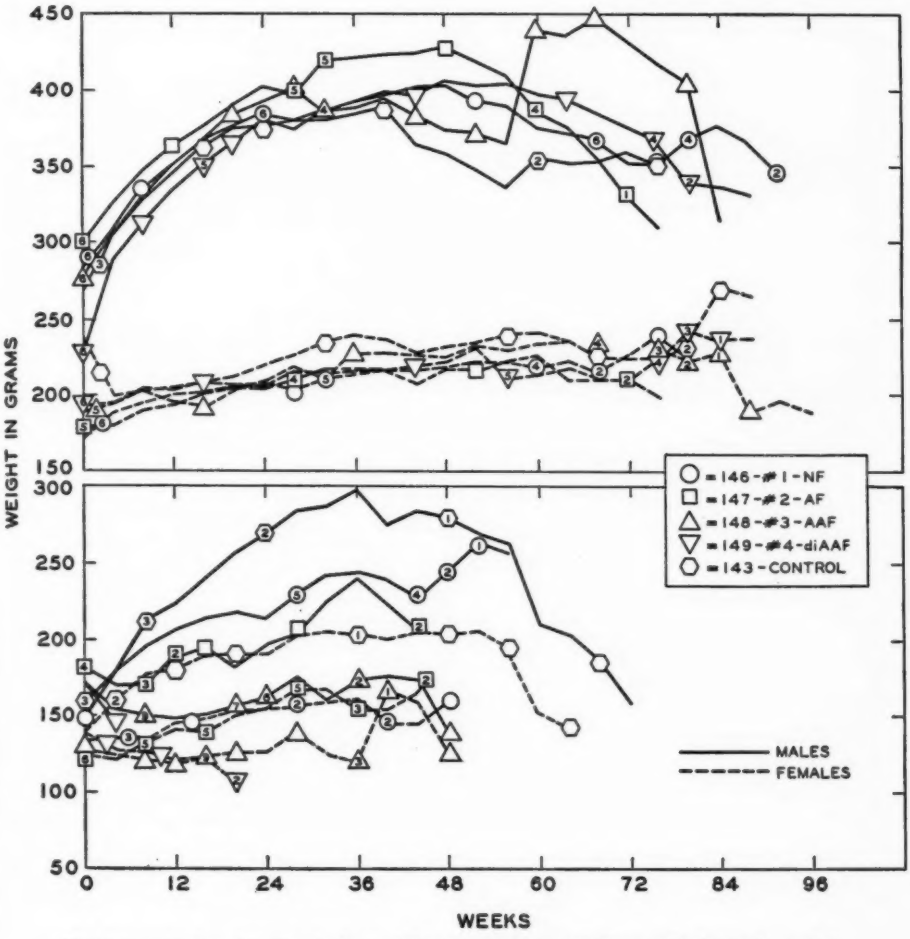
M=Minnesota strain. W=Wistar strain. B=Buffalo strain.

A striking difference between the painting and the ingestion experiments is brought out in the average estimated amount of carcinogen administered, for much more was administered by ingestion for each of the four compounds than was administered by skin application. This difference amounted to approximately ten times more NF, AF, and AAF, but only three times more diAAF (table 1). Less diAAF was ingested because of the very adverse effect on the health of the animals ingesting this compound, as well as the early development of hepatomas, which occurred in ten of the twelve rats in this group necropsied during the first 2 months of the experiment. These hepatomas were found after an average period of treatment of only 58 days as will be shown below.

While the average initial weights of the rats in the painting experiments were greater than for the ingestion experiments, there were other noteworthy differences. The females, for example, in both experiments increased very little in weight, except the female control animals ingesting the synthetic diet. Male rats ingesting diAAF lost weight rapidly, and survived approximately 4 weeks. Females receiving diAAF survived considerably longer than males, although only about half as long as females ingesting the other carcinogens. All groups of painted rats survived longer than rats ingesting the carcinogens in synthetic diets and also longer than control rats on the synthetic diet. Painted male rats gained weight during the first third of the experiment. Their weight fluctuated considerably, thereafter, but showed a downward trend for the most part (text-fig. 1). It is believed these body-weight differences could have been due to both the superior nutritive properties of the natural foodstuff diet fed the painted animals as well as to a lesser toxicity of the carcinogens when applied in smaller quantities to the skin.

It appeared worth while to study the distribution of tumors according to compound and method of administration, since all four compounds induced tumors by both routes. It was found that rats ingesting NF, AF, and AAF developed fewer tumors than rats painted with these compounds, even though approximately ten times more carcinogen was administered orally than by painting. Ingestion of diAAF, however, produced somewhat more tumors than did painting diAAF (table 2).

Multiple tumors were more frequent in the animals receiving AAF either by painting or by ingestion than in any other group, and the ingestion of AAF induced multiple tumors in a somewhat larger number of animals than did painting (table 2). Ingestion of AF and painting of diAAF induced essentially the same number of multiple tumors, 2.3 and 2.25 tumors per rat respectively, and at approximately the same number of different sites. Animals ingesting diAAF developed tumors in the shortest time, most of which were induced in the liver. These early tumors were diagnosed as hepatomas. They were small foci, clearly demarcated from surrounding liver tissue; cells of the tumors were enlarged and hyperchromatic, and the adjacent liver cells were compressed. These nodules were usually multiple, and no attempt was made to count all of them in every liver.



TEXT-FIGURE 1.—Average body weight of rats after painting with (upper), and ingestion of (lower), 2-nitro-, 2-amino-, 2-acetylamino-, and 2-diacetylaminofluorene.

TABLE 2.—*Total and multiple tumor incidence summarized according to carcinogen and method of administration*

	Fluorene derivative								Control	
	2-nitro		2-amino		2-acetyl-amino		2-diacetyl-amino			
	Painting	Oral	Painting	Oral	Painting	Oral	Painting	Oral	Painting	Oral
Total No. Tumor-bearing rats	6	2	7	5	7	12	9	12	0	0
Total No. Non-tumor-bearing rats	4	7	4	6	2	8	2	0	6	6
Total No. rats	10	9	11	11	9	20	11	12	6	6
Total No. tumors	8	2	9	9	15	23	15	12	0	0
Avg. No. tumors in tumor-bearing rats	1.3	1.0	1.3	1.8	2.1	1.9	1.7	1.0	0	0
Percent rats developing tumors	60	22	64	45	78	60	82	100	0	0
No. rats with multiple tumors	2	0	2	3	5	7	4	-----	-----	----
Avg. No. tumors per rat in multiple tumor-bearing rats	2	----	2	2.3	2.6	2.9	2.25	(*)	-----	----

*Only hepatomas were induced, which were almost always multiple, but they have been counted as one tumor per rat.

Painting with NF and AF produced two tumors in each of two rats. Each of two rats ingesting NF had a single tumor. These data, therefore, indicate that of the four compounds studied AAF administered either topically or orally produces the largest number of multiple tumors in the largest number of rats, an effect not considered in this case to be due to strain, because Minnesota albino strain rats received AAF topically, while Buffalo strain animals received the carcinogen orally.

A study of the site distribution of tumors produced by the four fluorene derivatives studied according to sex, compound, and method of administration, shows also that the 2–NF and the 2-diAAF, as well as the 2–AF, induce distant tumors of multiple sites in both sexes. The results seem to indicate differences in the locations of the induced tumors. Thus, liver tumors were not found following administration of 2–NF, and only two tumors of the mammary gland and one of the ear canal were found. AF induced the largest number of tumors of the mammae in female rats, equally distributed between ingestion and painting groups; but AF induced no tumors of the mammae in male rats. Four cholangiomas were also induced by AF, and three squamous-cell carcinomas of the skin (table 3). Only one of the latter three occurred near the site of painting. No other skin tumors were found, and no evidence of injury to the skin of painted rats was observed in any group.

AAF induced tumors at 10 different sites in this study, a larger number of sites than for any of the other 3 compounds, yet three fourths of all the tumors induced by AAF were tumors of the liver, mammary glands, and ear canal, and were about equally divided between male and female rats for each of the 3 sites. In males painted with AAF there were no hepatomas and no mammary tumors, but 1 ear tumor in a female was

TABLE 3.—*Distribution of tumors according to sex, compound, and method of administration*

Total tumors	Location and type	Fluorene																Control			
		2-nitro-				2-amino-				2-acetylamino-				2-diacetylamino-							
		Ingestion		Painting		Ingestion		Painting		Ingestion		Painting		Ingestion		Painting		Ingestion		Painting	
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
23	Liver (Hepatoma)									3	6			7	5		2				
7	Liver (Cholangioma)					2	1		1			1				2					
29	Mammary gland (Adenocarcinoma)	1			1	5		4			5	5				8					
1	Mammary gland (Sarcoma)											1									
11	Ear canal (Squamous-cell carcinoma)	1				1				3	4	1				1					
3	Skin (Squamous-cell carcinoma)								3												
5	Pituitary gland (Adenoma)				2				1			1	1								
3	Adrenal gland (Adenoma)				1								2								
1	Adrenal gland (Adenocarcinoma)											1									
2	Leukemia (Generalized)															1	1				
2	Lung (Lymphosarcoma)				2																
1	Subcutaneous (Fibroma)				1																
1	Salivary gland region (Anaplastic carcinoma)				1																
1	Thymus gland (Lymphoma)												1								
1	Kidney pelvis (Squamous-cell Carcinoma)									1											
1	Bladder (Squamous-cell carcinoma)										1										
1	Intestine (Reticulum-cell sarcoma)											1									
93	Total tumors by sex, compound, and route of administration	2	0	0	8	8	1	4	5	7	16	11	4	7	5	12	3	0	0	0	0
	Total No. rats	3	6	3	7	6	5	5	6	10	10	5	4	7	5	6	5	3	3	3	3
	Total tumors by sex and compound	♀ 2	♂ 8			♀ 12	♂ 6			♀ 18	♂ 20			♀ 19	♂ 8						
	Total No. tumors per male and female	0. 33	0. 53			1. 1	0. 54			1. 1	1. 5			1. 5	0. 8						
	Total No. tumors for each compound	10				18				38				27							
	No. tumors per rat according to compound	0. 53				0. 82				1. 21				1. 17							

found. Of the 7 other sites where tumors were induced by AAF 5 were in male, and 4 in female rats.

Twenty of the 23 tumors induced by diAAF were of the liver and mammary glands. Two of the remaining 3 were leukemia and the third was of the ear canal, a total of 4 sites. No tumors of the mammary gland were induced in male rats receiving diAAF.

The number of tumors [4] per rat summarized by combining all data for both methods of administration shows that per rat there were 0.53 tumors for NF, 0.82 for AF, 1.21 for AAF, and 1.17 for diAAF, from which it can be concluded that the relative carcinogenicity of NF was less than AF, which in turn was less than AAF or diAAF, the latter two being approximately equal in carcinogenic potency.

DISCUSSION

The experiments reported herein show that NF, AF, AAF, and diAAF are carcinogenic to the rat, inducing distant tumors of various sites, either after ingestion or after painting. Much smaller quantities (of the order of 1:10), however, were administered by painting than by feeding. Inasmuch as the painting was continued throughout the experimental period, whereas the feeding was carried for a maximum period of 23 weeks, the two are not easily comparable, but appear to be in agreement with other investigators who have suggested that the length of time during which AAF is administered is more important for demonstrating carcinogenicity than the total amount ingested (*3*).

The dosage level of ingested AAF was also found to be important. According to Wilson and DeEds (*10*), a level of 0.001 percent of AAF in the diet of rats for over 24 months failed to induce tumors. No data were given on amount of AAF ingested but an estimated daily food intake of 10 gm. per rat would amount to an AAF intake of 0.7 mg. per rat per week, which would amount to a total of 74 mg. per rat during the period of their experiment, an amount comparable to the amount administered in the above described painting experiments which did induce many tumors. It has been observed in this laboratory, in another experiment, that the feeding by stomach tube of 0.25 mg. per week per 100 gm. of rat, or an average of 0.47 mg. per rat per week for a period of 18 weeks, with an average total intake of 8.5 mg. AAF per rat, also resulted in only one microscopically verifiable tumor in 93 Buffalo strain rats. This one tumor could well have been of spontaneous origin. Increasing the dosage by 4 resulted in a tumor incidence of 5 in 106 rats plus several more animals with what may be called early precancerous changes.[5]

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[4] A microscopic diagnosis was made of each of these tumors by Dr. Thelma B. Dunn. Many were of unusual interest, particularly those involving the external auditory canal. It was decided, however, not to include a detailed report on the histopathology of these tumors in the present paper because their morphology and histogenesis, as well as many nonneoplastic pathological changes apparently also induced by these carcinogens, are still being investigated.

[5] Unpublished data.

These observations suggest that painting of AAF for tumor induction on a quantitative basis may be even more effective than oral administration. It raises the questions of the extent of the destruction of the carcinogen in the alimentary tract and whether or not only a small portion of ingested carcinogen actually is absorbed to function as an active carcinogen. Studies are now in progress in this laboratory with C^{14} and N^{15} labeled AAF designed to obtain more information on the absorption, distribution, and combination of this carcinogen in tissues of the rat as a part of studies on the mechanism of carcinogenesis.

The largest number of sites developing tumors occurred after administration of AAF. Although about 75 percent of the AAF tumors developed in the liver, mammary gland, or ear canal, tumors also developed in seven other sites. It is noteworthy that after AAF, liver and mammary gland tumors developed in about equal number in both sexes. Bielschowsky (*4*) has reported a low percentage (3 in 41 animals) of mammary tumors in male Wistar rats ingesting AAF. Cantarow *et al.* (*21*) and other investigators have not induced mammary cancer with AAF in male rats. Four Buffalo rats of 14 male rats receiving AAF developed mammary tumors, or 28 percent of the males, but no mammary tumors developed in males receiving diAAF. One likely explanation for the latter would be that hepatomas developed very early after feeding diAAF and the animals were autopsied before mammary tumors had time to appear, except that the 5 painted males out of the 10 males used in this experiment were painted with diAAF, and survived an average of 18 months. So here, also, there appears to be a difference in the action of the 2 compounds. The strain of rat is probably the most important factor in the developing of mammary tumors in males after AAF administration.

The development of a high incidence of early hepatomas noted in the data for Minnesota strain rats ingesting diAAF may be entirely a strain response, because a subsequent attempt to develop early hepatomas in Buffalo strain rats with diAAF given orally was unsuccessful.

The data of Dunning, Curtis, and Madsen (*7*) on females of five strains give further indication of the influence of strain on the site of tumor formation following the ingestion of AAF. Thus, in their experiments the Copenhagen strain was more susceptible to development of malignant tumors of the bladder but resistant to mammary tumor induction, whereas the Marshall strain was resistant to both bladder and mammary tumor induction but susceptible to malignant lesions of the liver.

The distribution of AAF or some conjugate of AAF throughout the tissues of the rat in concentrations sufficiently high to induce cancer (*22*) is one logical explanation for the production of many distant tumors. The differences in the types and locations of the distant tumors between the compounds studied in this and other experiments suggest some fundamental carcinogenic property that is altered by the nature of the substituent chemical groups on the fluorene molecule.

SUMMARY

The number and site of tumors induced in male and female rats after topical application and ingestion of 2-nitro-(NF), 2-amino-(AF), 2-acetylamino-(AAF), and 2-diacetylaminofluorene (diAAF) have been studied.

The compounds were applied in one series topically throughout an experimental period of 16 to 21 months, and in a feeding experiment the same compounds were ingested for a maximum of 23 weeks. All animals were observed until tumors developed or death occurred. Approximately one tenth as much NF, AF, and AAF, and one third as much diAAF was applied topically as was ingested. The painted animals survived much longer than the rats ingesting the compounds.

A total of 105 rats of 3 strains was included in the experiments. No tumors were found in the 12 control animals. Distant tumors were induced by all 4 compounds, and by both routes of administration as follows: NF induced 10 tumors in 19 rats, an average of 0.53 tumor per rat. AF induced 18 tumors in 22 rats, an average of 0.82 tumor per rat. AAF induced 38 tumors in 29 rats, an average of 1.21 tumors per rat. DiAAF induced 27 tumors in 23 rats, an average of 1.17 tumors per rat.

Tumors were induced at 10 sites by AAF. Seventy-five percent of these AAF tumors were of the liver, mammary gland, and ear canal. Tumors were induced in 8 sites by NF, 7 of which were in painted males. AF induced tumors at 5 sites and diAAF at 4 sites.

Liver tumors were induced as follows: By NF none, by AF only cholangiomas, and by AAF and diAAF mostly hepatomas, with a few cholangiomas.

Mammary tumors were induced by AAF in male Buffalo strain rats and in one male rat painted with NF. AF and diAAF did not induce mammary tumors in male rats.

Three skin tumors were induced by AF, only one of which was in a painted area. No skin tumors were induced by the other 3 compounds. Twenty-four of the 27 tumors induced by diAAF were of the liver and mammary gland; 2 were leukemia, and 1 was an ear tumor.

More tumors were induced by painting with NF than by its ingestion, but not by painting with AF, AAF, and diAAF, even though the topical application was for a much longer period.

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STUDIES ON THE EFFECTS *IN VITRO* OF ROENTGEN RADIATION ON THE BIOLOGICAL ACTIVITY OF THE AGENT OF CHICKEN TUMOR I (ROUS SARCOMA) [1]

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INTRODUCTION

The curve-relating dosage of X radiation or other ionizing radiation to the surviving activity in irradiated samples of biologically active agents, such as bacteria, viruses, and enzymes, may be of value in determining certain of their physical and chemical properties.[3] The following deals with studies of such relationships using the agent of chicken tumor I (Rous sarcoma) (*5*) and 186 kv X radiation.

The virus-like agent of chicken tumor I has been shown to be associated with the microsome [4] constituents of tumor tissue extracts (*8–14*), but all attempts to isolate it in pure form have thus far proved unsuccessful. The present investigation was for the purpose of determining whether the dose-survival curve for the agent of chicken tumor I obtained by irradiation with X radiation would differ appreciably in studies carried out with different types of impure and partially purified suspensions of the agent. More specifically, it was desired to know whether the various "impurities" which are present in tumor-tissue extracts, and which are separable to some degree by differential centrifugation, are capable of influencing the character of the dose-survival curve, or, whether a characteristic curve for the agent, *per se*, might be consistently obtained in spite of these "impurities." In the latter event, information regarding the size and structure of the agent might be gained through irradiation studies in spite of the lack of purity of the tumor-agent preparations. In the former event, information might be gained regarding the nature of the "impurities," which might aid in their eventual separation.

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[3] For references and discussions of the actions of radiations on living cells, including effects on bacteria, chromosomes, viruses and enzymes, see references *1–4*.

[4] The term "microsome" was employed by Claude (*6*) to designate one of the 3 major fractions of liver cell components that could be separated by differential centrifugation. It referred to that part of the liver suspension which is made up of particulate elements of submicroscopic size, ranging from 50 to 300 mμ, but which is sedimentable under centrifugation at relatively high speed. The term is now widely applied to that fraction in cell and tissue extracts in general which is not sedimented by centrifugation in an ordinary centrifuge, or at high speed for a short time, but which is sedimented in an ultracentrifuge under the conditions usually employed in the separation of viruses (*7*).

In the series of experiments reported herewith, the method of differential centrifugation was employed to produce different types of tumor-agent suspensions containing various relative proportions of the different tumor-tissue extractives that are separable by this method, and a determination was made of the dose-survival curve in each case.

MATERIALS AND METHODS

Preparation of tumor agent suspensions. See section headed "Experiments and Results" for the procedures employed in the individual experiments. All tumor-agent suspensions were prepared from tumor tissue that had been frozen and stored at about −50° C.

Irradiation of tumor agent suspensions. The X-ray machine employed consisted of a high voltage transformer and two self-rectifying water-cooled X-ray tubes, each housed in a protective shield with a bakelite window. The tubes were connected to the transformer in such a way that one alternation (half cycle) of the high voltage alternating current passed through one tube and the other alternation through the other tube. The tubes were placed in juxtaposition, with the focal spots approximately 21 cm. and the outside of the bakelite window approximately 8 cm. apart. The specimens to be irradiated were placed at mid-distance from the bakelite windows in a covered thin-walled celluloid dish of the petri type measuring 67 mm. in diameter and 22 mm. in depth, the thickness of the fluid specimen being not more than a few millimeters. Due to the bakelite windows, heating of the sample from visible or infrared radiation from the tube was negligible. Irradiations were carried out at room temperature, which did not vary more than 1° or 2° C during the course of an experiment. The tubes were operated at 186 kv peak and 20 m amps. Except for the inherent filtration of tube wall and bakelite window, which is equivalent to approximately 0.2 mm. of copper, no additional filtration was used. The diameter of the container was such that it was uniformly irradiated within the error of the dose measurements and the thickness of the fluid specimen such that the fluid dose was approximately uniform.

Dose-rate measurements were done with a Victoreen 5,000 r chamber. This chamber was calibrated before each run against a calibrated 100 r Victoreen chamber. The dose rate was determined before and usually also after each run. Dose rates for the different runs varied from 5,000 r per minute to 5,800 r per minute. The variation in dose rate for the different runs which extended over 1 year was due to slight variations in the distance of the two tubes from run to run and due to replacement of one tube. The error in the dose measurement is estimated to be ± 10 percent. All dose-rate measurements were done in air. As the fluid layer was only a few millimeters thick, scattering was negligible, and the fluid dose will correspond to the dose in air.

A control sample of each suspension was contained in a similar celluloid dish and was of the same volume as the treated sample in all instances. It was kept in an adjoining room, at a temperature that did not differ by more than 1 or 2 degrees C at any time from that of the irradiation room. In order that all factors other than irradiation would be comparable in the comparisons of the two materials, a subsample of the control was taken simultaneously with each subsampling of the test material, after specified accumulative time intervals of irradiation.

Each subsample of the irradiated suspension was compared directly with its corresponding control, with respect to potency, in a separate bioassay determination as described below. The volume of each subsample was 1.5 ml. The starting volume of each sample was 10 ml. in experiments 5 and 6, but was 20 ml. in all other experiments.

Bioassays. The bioassays were carried out as previously described (*15*, *16*). For each potency determination the irradiated material (unknown) was compared with its corresponding control (known) in a separate bioassay involving 40 chickens and 160 inoculation sites. The principles of the bioassay procedure have been described by Bliss and Marks (*17*). The biological response employed as a criterion of potency was the reciprocal of the latent period (*18*, *19*).

EXPERIMENTS AND RESULTS

Experiment 1

Procedures. Ten grams of frozen tumor tissue were allowed to thaw at room temperature and homogenized in 300 ml. of cold, 0.9 percent, NaCl solution by blending for 3 minutes in a Waring blendor. The homogenate was centrifuged at 2,300 g for 20 minutes in an angle head of an ordinary laboratory centrifuge to remove tissue debris. The supernatant was recovered and further clarified by centrifugation at 18,000 g for 5 minutes.[5] The fraction recovered following the latter centrifugation was the middle portion of the supernatant. The upper and lower portions were avoided to prevent re-inclusion of separated lipoid material or of loosely packed sedimented material.

The treatment given the extract was thought to be sufficient to remove most of the particulates larger than about 300 mμ in diameter (*6*). Retained in the extract were particulates belonging to the microsome fraction, including the active tumor agent, as well as other small particulates, and soluble tissue proteins and other constituents. Among the soluble tissue products retained were free hemoglobin, evidenced by a strong red color of the extract, and the mucoid substances that are characteristically present in tissues and extracts of chicken tumor I (*5*, *10*, *11*, *20*, *21*, *22*, *23*) [see also reference *24* for chemical studies on a similar substance from another chicken tumor].

[5] International Refrigerated Centrifuge Model PR-1, equipped with a multispeed attachment and a device which permitted the centrifuge head to come to rest gradually during deceleration.

Previous studies (*15*) had shown that the total nitrogen content of extracts such as that used in the present experiment is about 0.11 to 0.3 mg. per ml. The amount of soluble protein present in the extract was therefore assumed to be sufficiently high to rule out most of the indirect effects of irradiation [see reference (*1*), page 108] and the material was used in the experiment without further addition of soluble protein.

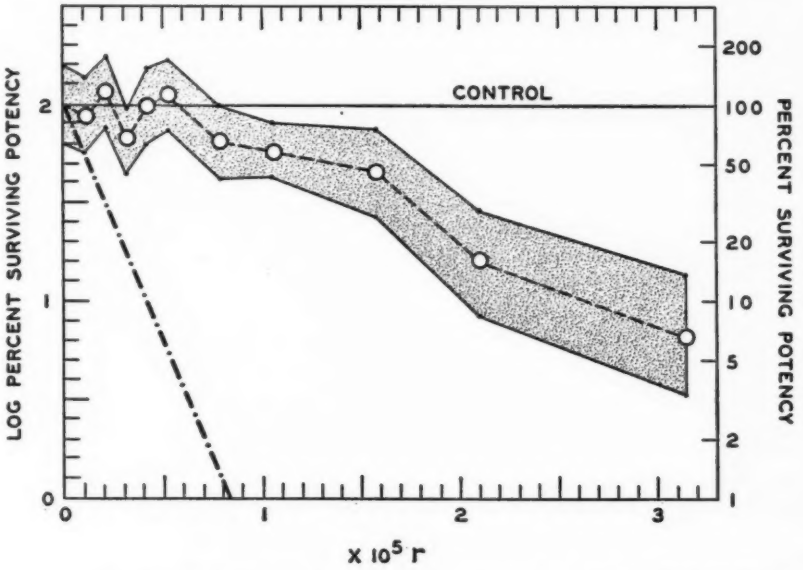
Results. After the first few minutes of irradiation the color of the extract was observed to have changed from red to amber. Later in the experiment, after about 20 minutes of irradiation, it was observed that the extract foamed easily on being drawn back and forth into the sampling pipette, a gentle mixing procedure that was used before taking each subsample. No such changes in color or in surface tension were noted in the control sample.

The results of the bioassays are shown in table 1 and in text-figure 1. The exact nature of the dose-survival curve for this type of material could not be determined decisively from the data of this single experiment, and therefore no attempt was made to derive the best fitting curve for this particular set of data. Whatever the true form of the best fitting curve might be, *i. e.*, whether convex upward, or slightly S-shaped, it is apparent that it has a flatter slope in the region of the lower than at the higher accumulated doses. Furthermore, the over-all slope of the curve

TABLE 1.—*Data of experiment 1 showing tumor-producing potency of a first extract of tumor tissue after various accumulative doses of X radiation*

Subsample number	Time of irradiation at the dose rate of 5,250 r per minute	Total dose (in units 10^4 r)	Surviving potency			
			Log percent potency	Standard error of log percent	Percent potency	Standard error of percent
	Minutes					
1	2	0.105	1.939	±0.192	87.0	+48.4 −31.1
2	4	.210	2.072	±.178	117.9	+59.9 −39.7
3	6	.315	1.807	±.162	64.1	+29.0 −20.0
4	8	.420	1.990	±.190	97.7	+53.5 −34.6
5	10	.525	2.049	±.182	112.0	+58.3 −38.3
6	15	.788	1.810	±.189	64.6	+35.3 −22.8
7	20	1.050	1.770	±.139	58.9	+22.3 −16.2
8	30	1.575	1.659	±.220	45.6	+30.0 −18.1
9	40	2.100	1.206	±.262	16.1	+13.3 −7.3
10	60	3.150	.836	±.315	6.9	+7.3 −3.5

is considerably flatter than that of the expected curve [6] for this agent, which is represented by the dash-dot line at the lower left of the figure.



TEXT-FIGURE 1.—Dose-survival curve of experiment 1 showing the tumor-producing potency remaining in a first extract of tumor tissue after the accumulated doses indicated.

The plotted points represent potencies relative to the corresponding controls. The shaded bands enclose limits of error corresponding to ± 1 standard error unit.

The straight, dash-dot line shows the expected relationship between dose and surviving potency (see text, and text-footnote 6).

[6] The nomogram published by Lea [reference (*1*), text-fig. 8] was used for determining the expected dose-survival curve for the agent of chicken tumor I. It shows the relationship between the diameter of biologically active particles and the 37 percent survival dose of various ionizing radiations. It was constructed on the assumptions that the active particles are spherical in shape and that the fundamental nature of the relationship between dose and surviving activity is exponential.

When surviving potency is considered on a log scale, as in the accompanying figures, the form of the curve is a straight line, and one of the parameters of the line is log 100 percent (2.0), surviving activity, at zero dose. Another parameter is determined by the size and density of the unit active particle. Where size and density are known, therefore, the expected irradiation dose-survival curve may be determined from the relationships described by Lea. It should be emphasized that the relationships are not presented by this author as precise, but as empirical approximations based upon the best experimental data available at the present time [see reference (*1*), p. 116 *et seq.*].

The 37 percent survival dose is generally employed in interpretations of irradiation data because it is the dose which corresponds to an average of a single ionization cluster per target. [For further discussion see reference (*1*), p. 72 *et seq.*]

In deriving the expected curve for the agent of chicken tumor I, the basic assumption was made that the unit active particle is spherical, the diameter of the particle was taken to be 70 m μ, which is the value estimated from ultracentrifugation studies (*10*, *11*, *13*, *25*) and the density was taken to be 1.3 (*10*, *11*, *13*).

The curve given by Lea [reference (*1*) text-fig. 8] for X radiation of effective wave length of 0.15A was used for determining the relationship between 37 percent dose and particle size. Although the effective wave length of the X radiation used in these experiments is of the order of 0.2A this difference in effective wave length will not alter the relationship to any appreciable extent because only inherent filtration was used. This can be readily seen from Lea's curves for radiations of different wave lengths and from the fact that the average ion density is roughly constant for the radiations from X-ray tubes of effective wave lengths from 0.15A to 0.4A.

Experiment 2

Procedures. Ten grams of tumor tissue were homogenized in 300 ml. of NaCl solution as described under experiment 1. The homogenate was centrifuged at 2,300 g for 20 minutes and the sediment was recovered for re-extraction. The supernatant, or first extract, was discarded. The tissue residue of the sedimented fraction amounted to about 1.5 grams. It was re-extracted by homogenization in 200 ml. of 0.1 molar phosphate buffer of pH 8. The second homogenate was clarified by centrifugation at 2,300 g to remove tissue residue, and the supernatant was recovered and subjected to further centrifugation at 18,000 g for 5 minutes. The supernatant from the latter centrifugation was recovered from the middle portion of the centrifuge tubes as described in the preceding experiment.

The recovered fraction represented a second extract of the type previously investigated with respect to potency as well as to relative purity of the agent on a nitrogen basis (*15*). It was grossly free of hemoglobin and mucoid substances, in addition to having a higher ratio of potency to nitrogen than first extracts such as that of the preceding experiment. Judging from the results of the previous studies (*15*), second extracts prepared in the manner described have a low total nitrogen content, about 0.002 to 0.008 mg. per ml., and the amount of soluble protein which they probably contain was not considered sufficiently high to rule out safely the indirect effects of irradiation. Normal rabbit serum was therefore added in an amount which made its concentration in the mixture 1.8 percent, by volume.

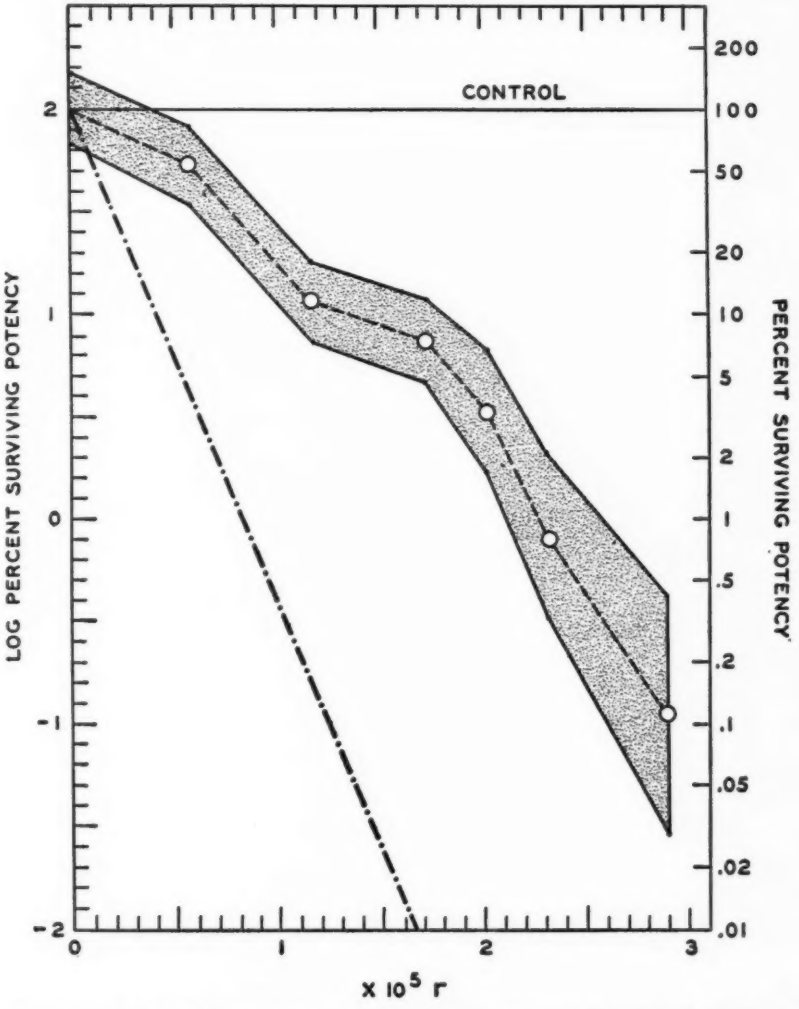
Results. No gross changes in the characteristics of the second extract were noted during irradiation. The results of the bioassays, shown in table 2 and in text-figure 2, suggest a curve which is convex upwards and

Table 2.—*Data of experiment 2 involving a second extract of tumor tissue. See legend of table 1*

Subsample number	Time of irradiation at the dose rate of 5,500 r per minute	Total dose (in units 10^3 r)	Surviving potency			
			Log percent potency	Standard error of log percent	Percent potency	Standard error of percent
	Minutes					
1	10	0.58	1.726	±0.186	53.2	+28.5 −18.5
2	20	1.16	1.051	±.195	11.2	+6.4 −4.1
3	30	1.74	.868	±.207	7.4	+4.5 −2.8
4	35	2.03	.521	±.302	3.3	+3.3 −1.7
5	40	2.32	−.096	±.401	.80	+1.22 −.48
6	50	2.90	−.953	±.581	.11	+.31 −.08
7	60	3.48	(1)		(1)	
8	70	4.06	(1)		(1)	
9	80	4.64	(1)		(1)	

[1] Potency too low to be measured quantitatively under the conditions employed for the bioassays.

the slope of which becomes steeper with the increase of accumulated dose. The curvature is somewhat similar to, but more pronounced than, that obtained with the first extract in the preceding experiment. The over-all slope of the dose-survival curve, though not as flat as that of experiment 1, is still considerably flatter than that of the expected curve [6] shown by the dash-dot line at the lower left of text-figure 2. It may, or may not, be of significance that the last four plotted points fall off on a straight line which has a slope very nearly the same as that of the expected curve.



TEXT-FIGURE 2.—Dose-survival curve of experiment 2 involving a second extract of tumor tissues. See legend of text-figure 1.

Experiment 3

Procedures. A first extract of tumor tissue was prepared in exactly the same manner as described under experiment 1 and 72 ml. of it were subjected to centrifugation at 18,000 g for 1 hour. The sediment was recovered and resuspended in 1.0 ml. of a buffer-serum solution composed of 0.05 molar phosphate buffer, of pH 8, and 2 percent normal rabbit serum, by volume. The concentrated suspension was kept over night in a refrigerator (0°–8° C) and on the following morning was diluted up to its original volume, 72 ml. It was then clarified by being subjected to three successive centrifugations at 18,000 g for 5 minutes. After each of the first two such centrifugations the supernatant was recovered by being poured off the closely packed pellets into new centrifuge tubes, but after the third centrifugation only the top ½ to ⅔ of the supernatant fluid was recovered from each of the six centrifuge tubes. This was accomplished by means of a transfer pipette. The bottom fractions of the fluid columns were avoided in order to prevent re-inclusion of loosely packed or incompletely sedimented aggregated material.

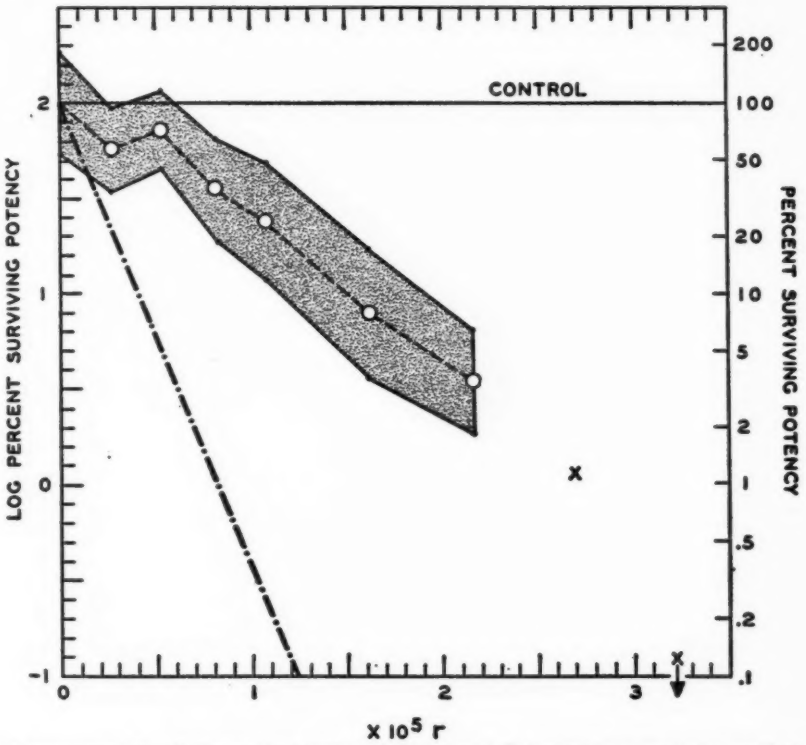
The recovered fraction represented a suspension of the microsome components derived from a first extract of tumor tissue by 1 complete cycle of differential centrifugation. Extra precautions had been taken to remove aggregates of material that had not been completely redispersed after having been sedimented during the long period of centrifugation at high speed. The suspending fluid contained a sufficient amount of soluble protein essentially to prevent indirect effects of the irradiation.

Table 3.—*Data of experiment 3 involving a partially purified microsome fraction derived from a first extract of tumor tissue. See legend for table 1*

Subsample number	Time of irradiation at the dose rate of 5,400 r per minute	Total dose (in units 10^3 r)	Surviving potency			
			Log percent potency	Standard error of log percent	Percent potency	Standard error of percent
	Minutes					
1	5	0. 27	1. 758	±0. 216	57. 2	+36. 9 −22. 4
2	10	. 54	1. 863	±. 194	73. 0	+41. 2 −26. 3
3	15	. 81	1. 550	±. 256	35. 5	+28. 5 −15. 8
4	20	1. 08	1. 381	±. 306	24. 0	+24. 5 −12. 1
5	30	1. 62	. 905	±. 340	8. 0	+9. 5 −4. 4
6	40	2. 16	[1] . 547	[1] ±. 265	[1] 3. 5	+3. 0 −1. 6
7	50	2. 70	[2] (. 06)	---	[2] (1. 1)	---
8	60	3. 24	[3]	---	[3]	---
9	80	4. 32	[3]	---	[3]	---

[1] The dose-response curves for control and irradiated materials showed a departure from parallelism which was significant at the 5 percent level. The bioassay results are therefore not definitely reliable.
[2] Approximate value, only; error not determinable. A sufficient number of test chickens to disturb the balance of the bioassay design died from tumors produced by the stronger control before the weaker irradiated material had had full opportunity to induce tumors (see reference *18*).
[3] See footnote 1 of table 2.

Results. The results are presented in table 3 and in text-figure 3. The plotted points of the figure would give a fairly good fit to the straight line that could be drawn through their weighted coordinate means and through log 100 percent potency (2.0) at zero dose; however, the slope of this line would, as indicated by the over-all trend of the data, be flatter than the expected slope and practically identical with that observed for the first half of the dose series in the preceding experiment. The expected curve [6] is shown by the dash-dot line. Since the first plotted point is lower than the trend of the remaining points, and since a straight line drawn through the remaining points would intersect the zero dose axis at a distance considerably above the control potency level (2.0 log percent), it is probable that the curve is flatter in slope at the lower doses as in the two preceding experiments. Furthermore, the extremely weak biological responses at the two highest doses of irradiation (table 3) indicate that the curve becomes very steep at these dose levels, the potency in both instances being less than could be measured quantitatively under the conditions employed for the bioassays.



TEXT-FIGURE 3.—Dose-survival curve of experiment 3 involving a partially purified microsome fraction derived from a first extract of tumor tissue. See legend of text-figure 1.

Experiment 4

Procedures. A second extract of tumor tissue was prepared as described under experiment 2 except that 0.9 percent NaCl solution instead of buffer was used for the second extraction, and there was only one centrifugation, at 2,300 g for 20 minutes, to remove tissue debris and large particulates. The middle fraction of the supernatant was recovered after the centrifugation at 2,300 g, and was subjected to further centrifugation at 18,000 g for 1 hour. The sedimented fraction was recovered and resuspended in its original concentration in buffer-serum solution composed of 0.05 molar phosphate buffer of pH 7, and normal rabbit serum, 2 percent by volume.

The recovered fraction represented the total microsome fraction derived from a second extract by high speed centrifugation. It contained all sedimentable particulates that had not been previously eliminated during centrifugation at 2,300 g, as well as much aggregated material that could not be redispersed by the mild procedure of being drawn back and forth into a capillary tipped pipette, after having been packed during the high speed centrifugation.[7] The latter procedure had been found satisfactory for the redispersion of certain viruses which can be purified by differential centrifugation [for discussions and references see (*7*)].

Results. The results of experiment 4 are shown in table 4 and in text-figure 4. As indicated in the last 3 rows of the table the bioassay results for the 3 highest doses were unsatisfactory because a sufficient number of test chickens to disturb the balance of the bioassay design died from tumors produced by the stronger control before the weaker irradiated materials

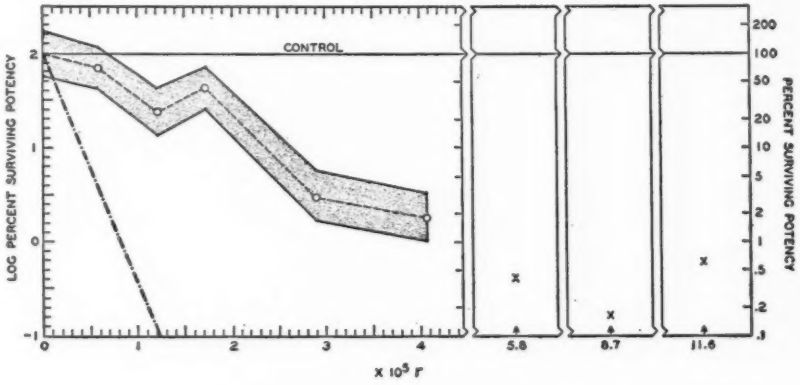
Table 4.—*Data of experiment 4 involving the total microsome fraction, including aggregates of unredispersed material, derived from a second extract of tumor tissue. See legend of table 1*

Subsample number	Time of irradiation at the dose rate of 5,800 r per minute	Total dose (in units 10^3 r)	Surviving potency			
			Log percent potency	Standard error of log percent	Percent potency	Standard error of percent
	Minutes					
1	10	0. 58	1. 849	±0. 211	70. 7	+44. 1 −27. 1
2	20	1. 16	[1] 1. 388	[1] ±. 250	[1] 24. 5	+19. 0 −10. 7
3	30	1. 74	1. 648	±. 218	44. 5	+29. 1 −17. 6
4	50	2. 90	. 491	±. 269	3. 1	+2. 7 −1. 4
5	70	4. 06	. 269	±. 265	1. 9	+1. 6 −. 8
6	100	5. 80	[2](−.38)	--------	[2] (. 42)	--------
7	150	8. 70	[2](−.79)	--------	[2] (. 16)	--------
8	200	11. 60	[2](−.22)	--------	[2] (. 60)	--------

[1] See footnote 1 of table 3.
[2] See footnote 2 of table 3.

[7] Evidence of the presence of large aggregates in materials of this type had been obtained in previous studies (unpublished) in which it was found that 80 percent or more of the biological activity could be removed with the sediment during clarification by centrifugation at 18,000 g for 5 minutes, or in an ordinary centrifuge.

had had full opportunity to induce tumors (*18*); however, it is significant that tumors did appear in nearly 50 percent of the inoculated sites, before such deaths began to occur, even with tumor-agent suspension that had been given a total dose of 1.16 million r. Although numerical values for potency are not definitely assignable in these instances, the results do indicate the continuation of a flat slope at higher accumulated doses. The extreme deviation of the observed dose-survival curve from the expected [6] is shown by its relation to the dash-dot line at the lower left of the figure. The points of this figure would fall within limits of experimental error about the smoothed curve that could be drawn through the points of experiment 1. The over-all slopes, and the displacements of the curves toward the higher doses, are therefore similar in experiments 1 and 4.



TEXT-FIGURE 4.—Dose-survival curve of experiment 4 involving the total microsome fraction, including aggregates of unredispersed material, derived from a second extract of tumor tissue. See legend of text-figure 1.

EXPERIMENT 5

Procedures. A second extract of tumor tissue was prepared as described under experiment 2 and the total microsome fraction was separated from it by centrifugation at 18,000 g for 1 hour, as described under experiment 4. The sedimented fraction was resuspended in distilled water and further fractionated to procure (A) active tumor agent in a highly dispersed state, and (B) active tumor agent in combination with aggregated constituents [7] which could not be readily redispersed by the mild procedure of being forced back and forth into a capillary tipped pipette. The subfractions were separated from each other by subjecting the total microsome fraction to centrifugation at 18,000 g for 5 minutes and recovering both the supernatant (A) and the sediment (B).

(A) The supernatant was further centrifuged three times in succession, at 18,000 g for 5 minutes, to remove any remaining large particulates or large particles of aggregated material. After each centrifugation the supernatant was recovered and transferred to a new tube for the next centrifugation, *etc.*, while the additional material sedimented during these runs was discarded. Following the third such centrifugation there was no visible sediment, and the recovered "supernatant" was subjected to centrifugation for 1 hour at 18,000 g. The sedimented fraction obtained after this long run was recovered and resuspended in buffer-serum solution consisting of 0.1 molar phosphate buffer, of pH 8, to which normal rabbit serum had been added in the amount of 2 percent, by volume. The resuspended sediment was clarified by centrifugation at 18,000 g for 5 minutes and the supernatant was recovered and employed as material A in the X radiation studies. It was a microsome fraction derived from a second extract of tumor tissue, and partially purified by 2 cycles of differential centrifugation which were interposed by extra clarification runs to remove undispersed material.

(B) The sediment procured during the original clarification of the total microsome fraction, by centrifugation at 18,000 g for 5 minutes, was resuspended in buffer-serum solution (see under A) and "washed" three times in succession by repeating these same processes of sedimentation and resuspension. After each sedimentation the supernatant was discarded and the sediment was resuspended in a new lot of buffer-serum solution. The sediment retained and resuspended following the third washing procedure was employed as material B in the irradiation studies.

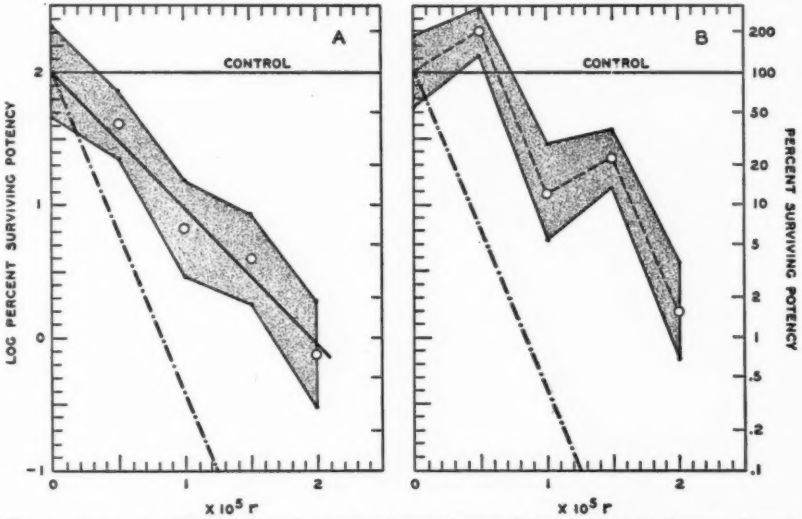
In order to insure the retention of sufficient amounts of biological activity in the 2 subfractions during the extensive procedures carried out to isolate the agent in the dispersed and in the aggregated states, the extract derived from 40 grams of tumor tissue was processed, and the concentrations of the subfractions in the suspending fluids were increased to approximately 10 times that in the original second extract. The relative concentrations of the constituents of materials A and B were exactly comparable in relation to the quantity of tumor tissue from which they were derived.

Results (A). The results of the investigations on material A are shown in table 5, section A, and in text-figure 5A. The plotted points of the figure give an excellent fit to the straight solid line drawn through their weighted coordinate means and through log 100 percent (2.0) potency at zero dose. This finding is consistent with the interpretation that, for preparations of the agent of chicken tumor I such as material A, there is an exponential relationship between dose and the surviving activity in irradiated samples. Whether the observed relationship represents the true curve for freely dispersed particles of the agent of chicken tumor I, or whether some factor is still present that modifies or interferes with the X radiation effects cannot be determined from the data of this single experiment. The observed results are much closer to the expected curve [6] shown by the dash-dot line of text-figure 5A, than in any of the preceding experiments, but the deviation from the expected curve is still significant as compared with

TABLE 5.—*Data of experiment 5 involving (A) dispersed, and (B) undispersed, or aggregated, subfractions of a microsome fraction derived from a second extract of tumor tissue. See legend of table 1*

Subfraction	Subsample	Time of irradiation at the dose rate of 5,000 r per minute	Total dose (in units 10^2 r)	Surviving potency			
				Log percent potency	Standard error of log percent	Percent potency	Standard error of percent
		Minutes					
A	1	10	0.50	1.606	±0.257	40.3	+32.6 −18.0
	2	20	1.00	.817	±.369	6.56	+8.78 −3.76
	3	30	1.50	.593	±.341	3.92	+4.67 −2.13
	4	40	2.00	−.130	±.388	.74	+1.07 −.44
B	1	10	.50	2.301	±.180	199.8	+102.8 −67.9
	2	20	1.00	1.079	±.372	12.0	+16.2 −6.9
	3	30	1.50	1.348	±.208	22.3	+13.7 −8.5
	4	40	2.00	.197	±.363	1.58	+2.06 −.89

the experimental error of the bioassays. It should be emphasized, however, that the relationship between particle diameter and 37 percent survival dose, on which the "expected" curve was based, is not presented by Lea (*1*) as exact, particularly in its relation to animal viruses, but as an empirical approximation based upon the best data available at the present time.



TEXT-FIGURE 5.—Dose-survival curves of experiment 5 involving (A) dispersed, and (B) undispersed subfractions of a microsome fraction derived from a second extract of tumor tissue. See legend of text-figure 1.

The straight, solid line of (A) passes through the weighted coordinate means of the experimental observations and through the control potency level (2.0 log percent, or 100 percent) at zero dose.

(B). The results obtained with material B are presented in table 5, section B, and in text-figure 5B. The plotted points are seen to be highly variable about any smooth, single phased, curve that could be drawn through them. This tendency was suggested in the data of experiment 4, which also involved active tumor agent in a state of aggregation or in association with insoluble constituents. It is possible therefore that the distribution of active agent among particles of aggregated material that binds or adsorbs it is heterogeneous, and that significant errors of subsampling are involved in bioassay studies on fractions of this type.

The general trend of the plotted points suggests a dose-survival curve that is flatter in slope at the lower than at the higher doses, and that is displaced toward the higher doses of X radiation as in the preceding experiments.

Experiment 6

Experiment 6 was of a preliminary nature and was for the purpose of determining whether aggregates composed of antigen-antibody complexes would have a characteristic effect on the nature of the dose-survival curve. In the absence of previous quantitative information regarding the most appropriate relative quantities of antigen and antiserum to be used, the experiment was planned more from the standpoint of obtaining orienting information on several possible outcomes rather than being designed most efficiently for studying specifically the nature of the dose-survival curve. Because of the extremely large difference in potency between the material subjected to irradiation and the reference material used for establishing relative potencies, the errors of the bioassays were excessive. The experiment is included in this report because the slope of the dose-survival curve happened to be essentially the same as that for the dispersed agent fraction (A) of the preceding experiment. Whether this is coincidental or whether it represents an important finding that may facilitate determination of the true curve for the agent of chicken tumor I and similar agents can be determined only by further investigations.

Procedures. The "antigen" used in the experiment was a highly active second extract of tumor tissue prepared as described under experiment 2 except that there was only 1 centrifugation, at 2,300 g for 20 minutes, to clarify it of tissue debris and larger particulates.

The antiserum was prepared in rabbits by intraperitoneal injections of materials similar to that described in experiment 4, *i. e.*, total microsome fractions derived from second extracts by centrifugation at 18,000 g for 1 hour. The serum was inactivated with respect to complement by heating at 56° C for 30 minutes.

The following materials were prepared and employed as described in the experiment:

Material I. To an aliquot of second extract normal rabbit serum was added in an amount that made its concentration in the mixture 2 percent, by volume. This material was designated as the "normal serum control." It was used as the standard of reference in bioassaying each of the other materials.

Material II. A sample of immune serum was irradiated for 40 minutes at a dose rate of 5,600 r per minute (total dose 2.24×10^5 r) and then added to an aliquot of second extract in an amount that made its concentration in the mixture 2 percent, by volume. The mixture was designated as the "previously irradiated immune serum control." It was for the purpose of testing the effect of radiation on the neutralizing ability of the immune serum.

Material III. To an aliquot of second extract, immune serum was added in an amount that made its concentration in the mixture 2 percent, by volume. A sample of the mixture was retained as an "immune serum control," which also served as the zero dose material in the irradiated series; it was designated as material III A.

Ten milliliters of the mixture were irradiated at a dose rate of 5,600 r per minute, and subsamples were withdrawn for bioassay after 20, 30, and 40 minutes. The successive subsamples of irradiated material were designated as materials IIIB, IIIC, and IIID, respectively. The immune serum control (IIIA) as well as the other controls (I and II) were kept at the same room temperature as the irradiated material during the period of X radiation.

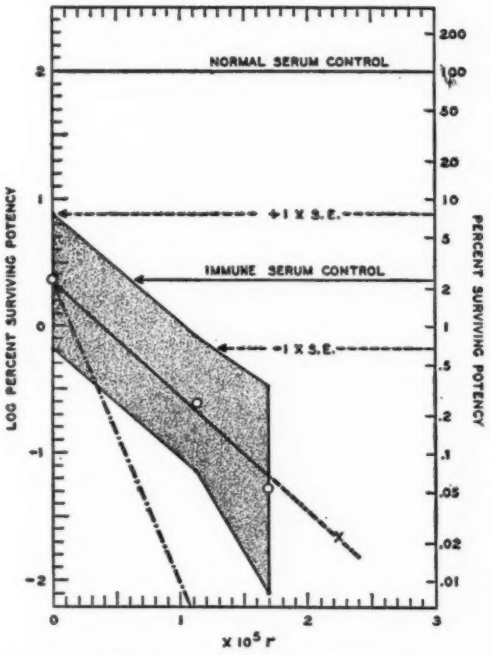
Results. The results of experiment 6 are shown in table 6 and in text-figure 6. The extent to which the biological activity was neutralized by the immune serum is indicated by a comparison between the potency of the immune serum control (material IIIA) and that of the normal serum control (material I). The former is seen from table 6 to be only about 2.3 percent as potent as the latter, and it is apparent that the added immune serum effectively neutralized about 97.7 percent of the tumor agent activity. Since the potency of the mixture containing previously irradiated immune serum (material II) was only about 2.2 percent of that of the normal serum control (material I), and was not significantly different from that of the mixture containing unirradiated immune serum (material IIIA), namely 2.3 percent, it can be concluded that 2.24×10^5 r of X radiation had no appreciable effect on the specific neutralizing antibodies of the immune serum.

The effects of irradiation on the mixture which contained immune serum and tumor agent (material III) is more readily seen in text-figure 6, in which the result on the unirradiated sample (IIIA) is plotted at zero dose. The result obtained after 40 minutes of irradiation (2.24×10^5 r), material IIID, was not entirely reliable because a sufficient number of the test chickens to disturb the balance of the bioassay design died from tumors produced by the stronger reference standard before the much weaker unknown had had full opportunity to produce tumors (see reference 18). The approximate value is included on the graph but it was not employed in deriving the weighted coordinate means for drawing the straight line through the plotted points. In drawing the line, the observed potency of the unirradiated sample was considered as the "true" potency for zero dose and the line was made to pass through this point. The level of reference for the irradiated material, *i. e.*, the immune serum control level,

TABLE 6.—*Data of experiment 6 involving active tumor agent in the presence of aggregates of antigen-antibody complexes. See legend of table 1*

Material	Description of material	Time of irradiation at the dose rate of 5,600 r per minute	Total dose (in units 10^4 r)	Potency			
				Log percent potency	Standard error of log percent	Percent potency	Standard error of percent
		Minutes					
I	Tumor agent plus normal rabbit serum ("normal serum control")		0	2.000		100	
II	Tumor agent plus previously irradiated immune rabbit serum ("previously irradiated immune serum control")		0	.338	±0.409	2.18	+3.40 −1.33
III	Tumor agent plus immune rabbit serum:						
(A)	Unirradiated ("Immune serum control")		0	.359	±.535	2.29	+5.57 −1.62
(B)	Irradiated	20	1.12	−.601	±.543	.251	+.626 −.179
(C)	Do	30	1.68	−1.264	±.810	.054	+.298 −.046
(D)	Do	40	2.24	[1] (−1.654)		[1] (.022)	

[1] See footnote 2 of table 3.



TEXT-FIGURE 6.—Dose-survival curve of experiment 6 involving active tumor agent in the presence of aggregates of antigen-antibody complexes. See legend of text-figure 1, and text.

is therefore subject to approximately the same experimental error as the other points. It is of interest to note that the line as drawn, has a slope which is practically identical with that of the preceding experiment obtained with a fraction (A) which contained the active tumor agent in a highly dispersed state (see text-figure 5A). The result is therefore contrary to that which would be expected if aggregates of antigen-antibody complexes were capable of altering the nature of the dose-survival curve in the manner observed in experiments 4, and 5B, and in which aggregates of another type were known to be involved. The immune serum employed in the experiment contained antibodies against all of the antigenic impurities of the microsome fraction in addition to the specific antibodies against the tumor agent. The results suggest that antibodies against these impurities may have rendered them ineffective in their ability to influence the slope of the dose-survival curve, possibly by preventing dissociation of their association with the agent following irradiation and during the dilution and vigorous mixing procedures which accompanied the bioassay procedure.

INTERPRETATIONS AND DISCUSSION

The results show that the nature of the dose-survival curve varied with the different types of tumor agent preparations, and that there was a

dependence of the irradiation result on the technique employed in preparing the agent suspension. The data of the several experiments cannot, therefore, be combined for arriving at a curve type which will represent the characteristics of the agent itself. Whether the curve for the partially purified and highly dispersed microsome fraction of experiment 5 (A) approximates closely the true curve for the agent of chicken tumor I, or whether interfering factors are still present remains to be proved. The fact that the preliminary result of experiment 6 gave a closely similar curve suggests that the approximation may be fairly close, and that a tentative interpretation of the results of these 2 experiments with respect to their implications regarding the agent particles may be of some value.

The 37 percent survival doses for materials A of experiment 5, and III of experiment 6 were found to be 4.37×10^4 and 4.83×10^4 r, respectively. The expected value, derived as described in footnote 6, was determined to be about 1.8×10^4 r. Using the nomogram of Lea [reference (*1*), text-fig. 8] and the assumptions described in footnote 6, the estimates of particle diameter were found from the above 37 percent doses to be about 49 mμ and about 46 mμ respectively. These values are within a factor of 2 of the estimate of 70 mμ obtained by other methods (see footnote 6) and therefore may be considered as consistent with it within the limits of accuracy of the irradiation method [see reference (*1*) page 116 *et seq.*].

If the value for size arrived at by the irradiation method were to be regarded as precise, then the discrepancy between the present and the previous estimates (see footnote 6) might be taken to indicate that all the substance of the unit particle is not radiosensitive, and that the irradiation method measures only the radiosensitive elements and not the total particle size, as is known to be the case with the larger animal viruses, such as vaccinia, and with bacteria (*1*). It is of interest to note in this connection that the electronmicrographs of chicken tumor I cells published by Claude, Porter, and Pickels (*25*) show small rounded submicroscopic particles, ranging from 67 to 85 mμ in diameter, which on close inspection seem to consist of darker appearing central areas surrounded by halos of lighter contrasting substance. The particles are thought by the authors to represent the tumor agent. The contrast between the central and peripheral areas was not mentioned by them. It is assumed that the measurements, 67 to 85 mμ reported represent the entire bodies and not the darker central areas, since, from the magnifications given, the diameters of the latter appear to fall between 40 and 50 mμ.

Assuming a spherical shape for both elements, the unit particle of the agent of chicken tumor I might then be regarded as having an over-all diameter of 70 mμ but containing an inner, radiosensitive, structure measuring 46 to 49 mμ in diameter, which is essential to the biological activity of the agent.

The dose-survival curves obtained in this series of experiments differed in one or both of two respects from the curve that would be expected on the assumption that the agent of chicken tumor I is a virus-like entity of the size and shape previously estimated or postulated.[6] These differ-

ences were: (a) departure of the curve from exponential (or, from a straight line when log surviving activity is plotted against dose), and (b) flattening of the over-all slope and displacement of the curve toward the higher doses. The latter shows that larger doses of X radiation were required to inactivate a given proportion of the agent than would be expected under ideal conditions, if the agent behaved like other known viruses in response to irradiation.

The irradiation data alone do not permit specific interpretation to be made regarding the exact nature of the "impurities," or of other factors which may have been responsible for, or contributed to, the deviations from the expected curve. Lea (*1*) has discussed various complicating factors that are capable of distorting an exponential survival curve, and one of them, namely aggregation, may be of significance in connection with the present findings.

Two of the experiments, 4 and 5B, were designed to test the effect on the dose-survival curve of the presence of large aggregates consisting of components of the microsome fraction which had not been completely redispersed following their sedimentation in an ultracentrifuge. That such aggregates are relatively large is evidenced by the fact that they can be sedimented in an ordinary centrifuge, or by centrifugation at high speed for a short time.[7] Whether the aggregates are composed chiefly of tumor agent, or whether they are composed chiefly of other microsome constituents, but include the active agent, is not known. Experiment 4 involved a suspension in which the tumor agent was present in both a relatively highly dispersed and an aggregated state. The deviation of the results from the expected curve was the greatest in this experiment. In experiment 5B, the aggregated fraction was separated from dispersed agent and "washed" repeatedly to remove loosely associated tumor agent. The results were highly variable about any smooth, single phased, trend but were definitely displaced from the expected curve toward the higher X radiation doses.

The closest approach to the expected dose-survival curve was obtained in experiment 5A. This experiment involved a microsome fraction which had been separated and partially purified by 2 cycles of differential centrifugation. Extra clarification runs (*i. e.*, high-speed centrifugation for a short time) had been employed to favor the more complete removal of aggregates of unredispersed material. The results of this and the above mentioned experiments are therefore consistent with the interpretation that relatively large aggregates composed of undispersed constituents of the microsome fraction are capable of distorting the dose-survival curve for the agent of chicken tumor I, as well as causing it to be displaced toward the higher X radiation doses.

Whether the factor of aggregation can account for the deviations observed in experiments 1, 2, and 3 is not known. In all 3 of these experiments the agent suspension subjected to irradiation had been clarified by procedures which were thought to be adequate for removing most of the large aggregates, *i. e.*, by centrifugation at 18,000 g for 5 minutes. In

experiment 3 the material had been clarified 3 times in succession by repeated centrifugation, the supernatant being recovered and transferred to a new centrifuge tube after each clarification run. If the deviations of the results from the expected curve are to be accounted for by aggregation in these instances, therefore it is necessary to assume either that (1) relatively small clumps of aggregated material are capable of producing the effect, or (2) that there were present in the extract much larger aggregates of some material of relatively low density which remained with the microsome constituents during centrifugation and which were in some way combined or associated with appreciable amounts of active tumor agent. It may be recalled in the latter connection that the microsome fraction derived from extracts of chicken tumor I contains from 24 to 48 percent of extractable lipoid material (*27*, *28*, *29*). No evidence was obtained that aggregates of antigen-antibody complexes are capable of distorting the dose-survival curve.

Three other factors that may have contributed to, or have been responsible for, the deviations and distortions of the dose-survival curves are:

(1) A differential effect of the radiation on the integrity of the tumor agent and on some associated substance capable of inhibiting or "masking" the tumor-producing activity to some degree. Thus, tumor agent in a "masked" state would not have contributed to the control response, and the release of active agent during irradiation, proceeding simultaneously with reduction in total agent due to radiation, would result in a dose-survival curve which would appear to have a flatter slope than expected if the increments of fall in activity are measured from the potency level of the control (unirradiated) suspension.

(2) Reversibility of the changes caused by radiation. Recent discoveries have shown that bacteriophage particles which have been made inactive by ultraviolet light (*30*, *31*) or X radiation (*32*) may be reactivated under certain conditions. Thus, Luria and Dulbecco (*30*) using ultraviolet light, and Watson (*32*) using X radiation have found that when a susceptible bacterium is infected with multiple "inactive" phage particles, combinations resembling "genetic recombination" may take place between the "inactive" particles to yield active or "reactivated" phage. This was referred to as "multiplicity reactivation." It was further demonstrated by Dulbecco (*31*) that ultraviolet inactivated phage particles could also be reactivated by visible light. Whether the agent of chicken tumor I is susceptible to reactivation after being inactivated by X radiation, or whether the conditions of the present experiments are favorable for such reactions to take place are not known, but this phenomenon should be considered as a possible mechanism which could account for displacement of the dose-survival curves toward the higher doses in the present series of experiments.

(3) Differences in initial concentration of tumor agent in the suspensions subjected to irradiation. Although differences in the initial con-

centration of tumor agent would not be expected to alter the nature of the dose-percentage survival curve resulting from the direct effects of X radiation, the absolute concentration of agent might be of importance in determining the curve if the amount of protective protein were not sufficiently high to rule out essential indirect radiation effects, such as those due to H and O H radicles, or if factors which distort the curve, such as those described above, are dependent upon concentration.

The experiments were carried out at different times with different batches of tumor tissue, and it was not possible to make a direct determination of the relative potencies of the various materials used in the different experiments. It is possible, however, to make a rough estimate of the order of their relative potencies from previous experience with similar materials in this laboratory.

The first extract employed in experiment 1 was undoubtedly the most potent material studied, and the materials investigated in experiments 2, 3, and 4 would not be expected to be weaker by more than 2 to 8 fold. The agent-immune serum mixture employed in experiment 6 was probably only about 1 percent as potent as the first extract of experiment 1, but it was nevertheless still highly active biologically. The relative potencies of the materials employed in experiments 5A and 5B are more difficult to judge from previous experience because of wide variations among different batches of tumor tissue extracts in the relative amount of biological activity which is lost during differential centrifugation. It is probable that both materials were less than 10 percent but more than 1 percent as potent as the first extract of experiment 1.

SUMMARY

The relation between dose of X radiation and the surviving activity in irradiated samples of the agent of chicken tumor I (Rous sarcoma) was determined in 6 experiments carried out with quantitative bioassay techniques.

The characteristics of the dose-survival curve were found to vary with the method of preparation, and with the relative "purity," of the tumor-agent suspension subjected to irradiation. The suspensions investigated ranged from crude extracts of tumor tissue to a microsome fraction which had been separated and partially "purified" by 2 cycles of differential centrifugation.

The diameter of the radiosensitive structure of unit particles of chicken tumor I agent is estimated from the best results of this series of experiments to be 46–49 mμ. The possible implications of this finding with respect to internal structure of the unit particles are discussed.

Evidence is presented that aggregation of constituents of the microsome fraction, with which the active tumor agent is associated may be responsible, at least in part, for the variations among dose-survival curves observed in the different experiments.

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FORMATION OF TYROSINE CRYSTALS FROM LEUKOCYTES AND VARIOUS NORMAL AND PATHOLOGICAL TISSUES BY MEANS OF A SYNTHETIC DETERGENT [1] [2]

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Recently, Ayres (*1*) and Ayres and Starkey (*2*) demonstrated that Charcot-Leyden crystals would form from eosinophils on exposure of the eosinophils to a synthetic detergent: Aerosol MA [3] (dihexyl sodium sulfosuccinate). During this work, tyrosine crystals formed in the buffy layer of blood in two cases of chronic myelogenous leukemia and in one case of multiple myeloma. At that time, it was considered that the formation of the tyrosine crystals might be an indication of malignant disease or at least of destructive disease. Experimental work, herein reported, proved this hypothesis false. The purpose of this paper is to report various aspects concerning the formation of tyrosine crystals by the action of a synthetic detergent on leukocytes. The problem was approached from the following standpoints: (1) to determine whether there was a difference in tyrosine-crystal formation in the buffy layer (leukocytes) of patients with malignant disease, in patients with nonneoplastic but destructive diseases, and in apparently normal persons; (2) to determine if tyrosine crystals were formed from leukocytes, or from the admixture of plasma and red cells in the buffy layer; (3) to determine if tyrosine crystals would form from leukocytes in the buffy layer without the action of a synthetic detergent; (4) to determine if tyrosine crystals would form in cancerous and noncancerous tissue by the action of a synthetic detergent; and (5) to confirm the identity of the tyrosine crystals by chemical studies.

TECHNIQUE

Four and one-half cubic centimeters of blood obtained by venipuncture was mixed with 0.5 cc. of a 3.8 percent solution of sodium citrate. The blood was centrifuged, and the buffy layer removed by means of a capillary pipette. One drop of this buffy layer was placed on a microslide. The drop was covered with a coverslip containing Aerosol MA. Aerosol MA was rubbed over the entire surface of the coverslip. The waxlike nature of

[1] Received for publication, February 14, 1950.

[2] The opinions and views set forth are those of the author and not to be considered as reflecting the policies of the Navy Department.

[3] American Cyanamid Corporation.

the compound permits it to adhere readily to the surface. The coverslips were rimmed with petrolatum to prevent drying, and the preparation was kept at room temperature. According to Beeler (*3*), Aerosol MA is dihexyl sodium sulfosuccinate. It is a homologue of Aerosol AY, Aerosol IB, and Aerosol OT, and is a commercially pure, white, waxlike compound that is somewhat hygroscopic. The solubility is 34.3 gm. per 100 cc. of water at 25° C. It has been used as a wetting agent, an emulsifying agent, and a detergent.

The preparations were observed daily for a period of 14 days, unless otherwise stated, and the presence or absence of tyrosine crystals was recorded.

The crystals appeared in the form of clumps of needles, with a constriction in the center (figs. 1 and 2). Some of the crystals were fan-shaped. The crystals were anisotropic and averaged 200 microns in length and 1 micron in width. They varied in number from a single crystal in an entire preparation to approximately 20 in a low-power microscopic field.

EXPERIMENTAL

Experiment 1: To determine incidence of tyrosine crystals in Aerosol MA-preparations of buffy layer in patients with malignant disease.

Preparations of buffy coat and Aerosol MA were made according to the described technique. Fifty-four patients with proved malignant disease were studied. In all cases the diagnosis was established by biopsy or at autopsy. The results are shown in table 1.

In 30 of the 54 patients, or 55 percent, tyrosine crystals developed. Most of the preparations became positive on the fifth day.

Experiment 2: Tyrosine-crystal formation in Aerosol MA-buffy-coat preparations in patients with destructive diseases.

Thirty-three patients were studied with diseases in which there was considerable breakdown of tissue, usually incident to infection. It will be noted from table 2, that 19, or 57 percent, of the patients were positive for tyrosine crystals.

Experiment 3: Formation of tyrosine crystals in Aerosol MA-buffy-layer preparations of normal persons.

Preparations were made according to the described technique on 80 normal students attending a laboratory technicians' course at the Naval Medical School. In this series 43, or 54 percent, of the preparations showed tyrosine crystals.

Experiment 4: Demonstration of tyrosine crystals in buffy layer but not in red blood cells or in plasma on exposure to Aerosol MA.

In the separation of buffy layer there is an admixture of plasma and red blood cells. To rule out the development of tyrosine crystals from red blood cells and plasma, the following experiment was devised. The blood of a mixed group of 150 patients and normal persons was obtained, citrated, and centrifuged according to the previously described technique. One drop each of plasma, buffy layer, and red-blood-cell suspension was

TABLE 1.—*Incidence of tyrosine crystals in Aerosol MA-buffy layer preparations in patients with proved malignant disease*

Case No.	Diagnosis	Days													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	Chronic myelogenous leukemia	−	−	−	−	+	+	+	+	+	+	+	+	+	+
2	Chronic myelogenous leukemia	−	−	−	−	+	+	+	+	+	+	+	+	+	+
3	Multiple myeloma	−	−	−	−	+	+	+	+	+	+	+	+	+	+
4	Adenocarcinoma of stomach	−	−	−	−	−	−	+	+	+	+	+	+	+	+
5	Adenocarcinoma of stomach	−	−	−	−	−	−	−	−	−	−	−	−	−	−
6	Hodgkin's lymphoma	−	−	−	−	−	−	−	−	−	−	−	−	−	−
8	Hodgkin's lymphoma	−	−	−	−	−	−	−	−	−	−	−	−	−	−
10	Lymphosarcoma	−	−	−	−	−	−	−	−	−	−	−	−	−	+
13	Carcinoma of breast	−	−	−	−	−	−	−	−	−	−	−	−	−	+
14	Carcinoma of breast	−	−	−	−	+	+	+	+	+	+	+	+	+	+
15	Carcinoma of cervix	−	−	−	−	+	+	+	+	+	+	+	+	+	+
16	Carcinoma of breast	−	−	−	−	−	−	−	−	−	−	−	−	−	−
17	Lymphoepithelioma of nasopharynx	−	−	−	−	−	−	−	−	−	−	−	−	−	−
18	Epidermoid carcinoma of palate	−	−	−	−	−	−	−	−	−	−	−	−	−	−
19	Hodgkin's lymphoma	−	−	−	−	−	−	−	−	−	−	−	−	−	−
20	Malignant melanoma	−	−	−	−	−	−	−	−	−	−	−	−	−	−
21	Sarcoma of antrum	−	−	−	−	+	+	+	+	+	+	+	+	+	+
22	Epidermoid carcinoma	−	−	−	−	−	−	−	−	−	−	−	−	−	−
23	Transitional cell carcinoma of bladder	−	−	−	−	−	−	−	−	−	−	−	−	−	−
24	Adenocarcinoma of uterus	−	−	−	−	−	−	−	−	−	−	−	−	−	−
25	Adenocarcinoma of stomach	−	−	−	−	−	−	−	−	−	−	−	−	+	+
26	Epidermoid carcinoma of ear	−	−	−	−	−	−	−	−	−	−	−	−	+	+
27	Epidermoid carcinoma of tongue	−	−	−	−	−	−	−	−	−	−	−	−	−	−
29	Epidermoid carcinoma of pharynx	−	−	−	−	−	−	−	−	+	+	+	+	+	+
30	Epidermoid carcinoma of cervix	−	−	−	−	−	−	−	−	−	−	−	−	+	+
31	Scirrhous carcinoma of colon	−	−	−	−	−	−	−	−	−	−	−	−	−	−
34	Metastatic carcinoma to bone	−	−	−	−	−	−	−	+	+	+	+	+	+	+
66	Carcinoma of lung	−	−	−	−	−	−	−	+	+	+	+	+	+	+
83	Monocytic leukemia	−	−	−	−	−	−	−	−	+	+	+	+	+	+
84	Carcinoma of ovary	−	−	−	−	−	−	−	−	−	−	−	−	−	−
100	Carcinoma of lung	−	−	−	−	−	−	−	−	+	+	+	+	+	+
120	Carcinoma of cervix	−	−	+	+	+	+	+	+	+	+	+	+	+	+
121	Carcinoma of stomach	−	−	−	−	−	−	−	−	−	−	−	−	−	−
122	Carcinoma of colon	−	−	−	−	−	−	−	+	+	+	+	+	+	+

TABLE 1.—*Incidence of tyrosine crystals in Aerosol MA-buffy layer preparations in patients with proved malignant disease*

Case No.	Diagnosis	Days													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
131	Carcinoma of lung	−	−	−	−	−	−	−	−	−	−	−	+	+	+
132	Glioblastoma multiforme	−	−	−	−	−	−	−	−	−	−	−	−	−	−
160	Malignant tumor, brain	−	−	−	−	−	−	−	−	−	−	−	−	−	−
168	Carcinoma of larynx	−	−	−	−	−	−	+	+	+	+	+	+	+	+
169	Carcinoma of stomach	−	−	−	−	−	−	−	−	−	−	−	−	−	−
171	Carcinoma of bladder	−	−	−	−	−	−	+	+	+	+	+	+	+	+
172	Carcinoma of prostate	−	−	−	−	−	−	−	−	−	−	−	−	−	−
196	Lymphoepithelioma	−	−	−	−	−	−	+	+	+	+	+	+	+	+
197	Carcinoma of prostate	−	−	−	−	+	+	+	+	+	+	+	+	+	+
198	Carcinoma of bile duct	−	−	−	−	−	−	+	+	+	+	+	+	+	+
203	Bronchiogenic carcinoma	−	−	−	−	−	−	−	−	−	−	−	−	−	−
241	Carcinoma of prostate	−	+	+	+	+	+	+	+	+	+	+	+	+	+
242	Carcinoma of cervix	−	−	−	+	+	+	+	+	+	+	+	+	+	+
243	Carcinoma of colon	−	−	−	+	+	+	+	+	+	+	+	+	+	+
245	Carcinoma of colon	−	−	+	+	+	+	+	+	+	+	+	+	+	+
246	Carcinoma of breast	−	−	−	+	+	+	+	+	+	+	+	+	+	+
247	Carcinoma of prostate	−	−	−	−	−	−	−	−	−	−	−	−	−	−
248	Carcinoma of cervix	−	−	−	−	−	−	−	−	−	−	−	−	−	−
249	Hypernephroma	−	−	−	−	−	−	−	−	−	−	−	−	−	−
250	Carcinoma of stomach	−	−	−	−	−	−	−	−	−	−	−	−	−	−

TABLE 2.—*Incidence of tyrosine crystals in Aerosol MA-buffy-layer preparations in patients with destructive nonneoplastic diseases*

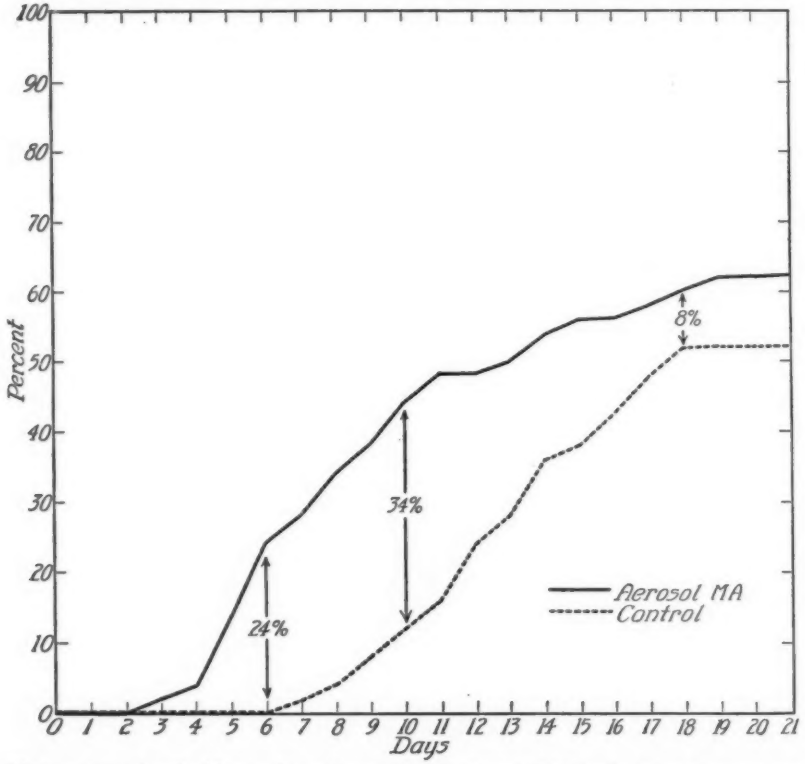
Case No.	Diagnosis	Days													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
37	Severe chronic bronchitis, bronchiectasis	−	−	−	−	+	+	+	+	+	+	+	+	+	+
38	Severe emphysema	−	−	−	−	−	−	−	−	−	−	+	+	+	+
39	Severe bronchitis, recurrent attack of pneumonia	−	−	−	−	−	−	−	−	−	−	−	−	−	−
40	Severe bronchitis, recurrent attack of pneumonia	−	−	−	−	−	−	−	−	−	−	−	−	−	−
41	Chronic bronchial asthma	−	−	−	−	−	−	−	−	−	+	+	+	+	+
42	Aspergillosis of lungs (?)	−	−	−	−	−	−	−	−	−	−	+	+	+	+
44	Chronic bronchial asthma	−	−	−	−	−	−	−	−	−	−	+	+	+	+
46	Rheumatoid arthritis, back	−	−	−	−	−	−	−	−	−	−	−	−	+	+
52	Chronic osteomyelitis	−	−	−	−	−	−	−	−	−	−	−	−	+	+
54	Coronary thrombosis	−	−	−	−	−	−	−	−	−	−	−	−	+	+
57	Cerebral hemorrhage	−	−	−	−	−	−	−	−	−	−	−	−	+	+
60	Osteomyelitis, left tibia	−	−	−	−	−	−	−	−	+	+	+	+	+	+
61	Coronary thrombosis	−	−	−	−	−	−	−	−	−	−	−	−	+	+
68	Degenerative arthritis	−	−	−	−	−	−	−	−	−	−	−	+	+	+
69	Rheumatic myocarditis	−	−	−	−	−	−	−	−	−	−	−	−	+	+
74	Lobar pneumonia	−	−	−	−	−	−	−	−	−	−	−	−	+	+
99	Bronchial asthma	−	−	−	−	−	−	−	−	−	−	−	−	+	+
101	Chronic arthritis, knee	−	−	−	−	−	−	−	−	−	−	−	−	+	+
122	Pulmonary tuberculosis, early	−	−	−	−	−	−	+	+	+	+	+	+	+	+
125	Pulmonary tuberculosis, early	−	−	−	−	−	−	−	+	+	+	+	+	+	+
127	Pulmonary tuberculosis, early	−	−	−	−	−	−	−	−	−	−	−	−	+	+
128	Pulmonary tuberculosis, moderately advanced	−	−	−	−	−	−	−	−	−	−	−	+	+	+
129	Pulmonary tuberculosis, early	−	−	−	−	−	−	−	−	−	−	−	+	+	+
155	Pulmonary tuberculosis	−	−	−	−	−	−	−	−	+	+	+	+	+	+
163	Pelvic inflammatory disease	−	−	−	−	−	−	−	−	+	+	+	+	+	+
165	Bronchial asthma	−	−	−	−	−	−	−	−	−	−	−	−	−	−
166	Cirrhosis of liver	−	−	−	−	−	−	−	−	−	−	−	−	−	−
176	Chronic osteomyelitis	−	−	−	−	−	−	−	−	−	−	−	−	−	−
177	Otitis media	−	−	−	−	−	−	−	−	−	−	+	+	+	+
179	Arthritis, generalized	−	−	−	−	−	−	−	−	−	−	−	−	−	−
183	Arthritis of back	−	−	−	−	−	−	−	−	−	−	+	+	+	+
180	Chronic arthritis	−	−	−	−	+	+	+	+	+	+	+	+	+	+
244	Abscess of buttock	−	−	−	−	−	−	−	−	+	+	+	+	+	+

placed on separate slides. Each was covered with a coverslip containing a film of Aerosol MA. Observations were made daily for 14 days, and the presence or absence of tyrosine crystals in each of the 3 preparations was recorded. In the buffy-layer preparations, 40 percent were positive for tyrosine crystals, while in the plasma and red-blood-cell preparations, no cases were positive for tyrosine crystals.

Experiment 5: Control studies to determine if tyrosine crystals form in buffy layer on standing without the addition of Aerosol MA.

In the preceding experiments no control studies were done to determine if Aerosol MA was responsible for the tyrosine-crystal formation. In this study 50 routine hospital admissions were selected, and Aerosol MA-buffy-layer preparations were made as previously described. In addition, a drop of buffy coat was placed on a slide, and covered with a coverslip not containing Aerosol MA. Both preparations were examined daily for a period of 21 days. The results are shown in text-figure 1.

It will be noted that tyrosine crystals were first formed on the third day, and were present in 24 percent of the preparations on the sixth day. In the control preparations, crystals were first noted on the seventh day, 4



TEXT-FIGURE 1.—Incidence of tyrosine crystals in Aerosol MA-buffy-layer preparations and in buffy-layer preparations without Aerosol MA.

days after the crystals were first noted in the experimental preparations. The maximal difference in the experimental and control preparations was 34 percent and occurred on the tenth day. The minimal difference was 8 percent, and occurred on the eighteenth day. Tyrosine crystals were found in 62 percent of the experimental preparations and in 52 percent of the control preparations.

Experiment 6: Formation of tyrosine and other crystals in malignant tissue and nonneoplastic tissue by means of Aerosol MA.

Malignant tissue was scraped with a scalpel, and the tissue thus obtained placed on a slide. A coverslip containing a thin film of Aerosol MA was placed over the material. The coverslip was rimmed with vaseline to prevent drying. Observations were made daily for crystals. In 3 cases out of 10, tyrosine crystals were obtained. These cases were lymphosarcoma, lymphoblastic type (A49–281), carcinoma of pancreas (A49–280), and transitional-cell carcinoma of the bladder (49–386B). Much to our surprise, other crystals formed in addition to tyrosine crystals. For example, crystals obtained from transitional-cell carcinoma of the bladder (49–396B) are shown in figure 5. Crystals from carcinoma of the colon (A49–223) are shown in figure 4. In a patient with chronic myelogenous leukemia (A49–251), in addition to tyrosine crystals, crystals similar to those of phenylalanine formed (fig. 3).

Nonneoplastic tissue when exposed to Aerosol MA by the technique described will also form crystals. For example, Charcot-Leyden crystals and tyrosine crystals were obtained from the excised spleen of a patient with thrombocytopenic purpura (49–1971), another type of crystal formed in minced tissue of normal thyroid gland (fig. 8). These crystals obtained from neoplastic and nonneoplastic tissue require further study to determine their nature and significance. Also, in preparations containing human red cells, hemoglobin crystals formed (fig. 6). When Aerosol MA was mixed with the hemoglobin of the dog, hemoglobin crystals also formed (fig. 7).

Determination of the Chemical Nature of the Tyrosine Crystals

Although tyrosine crystals have a rather striking appearance in that they are in clumps of needles, with a constriction in the center resembling a sheaf of wheat, or are in fan-shaped clusters of needles, they may be easily confused with other crystals. According to Todd and Sanford (*4*) the shape of tyrosine crystals may be similar to, or identical with, the crystals of calcium phosphate or ammonium biurate.

A large number of crystals for chemical studies were obtained as follows: Approximately 2,000 cc. of whole blood was obtained from the body of a patient who had died of chronic myelogenous leukemia. This patient had terminally a total white-blood-cell count of approximately 500,000 white blood cells per cu.mm. and 900,000 red blood cells per cu.mm. This blood was centrifuged, and a large quantity of buffy layer obtained. To 20 cc. of this buffy layer 1 gram of Aerosol MA was added. On 3-days' standing at room temperature a large number, 20 per low-power micro-

scopic field, of tyrosine crystals formed (fig. 1). These crystals were compared with known tyrosine crystals (*l*-tyrosine Merck) recrystallized from hot water to obtain the needle form. Under the polarizing microscope, both crystals had the same appearance, both were anisotropic, and both had the same optical sign.

Chemical determinations were done on this material obtained from the leukemic blood by Lt. E. E. Ozburn, MSC USN. He was able to obtain a positive test for tyrosine by a method originated by Gerngross *et al.* (*5*), as described by Feigl (*6*). In this test the material is exposed to α-nitroso-β-naphthol and yellow nitric acid; a purple color develops in the presence of tyrosine. An intense purple color was obtained from the leukemic buffy layer Aerosol MA mixture. A similar reaction was obtained with *l*-tyrosine (Merck). The material was deproteinized by trichloracetic acid, and the tests were done on the filtrate.

Control mixtures of Aerosol MA and red blood cells, which had been permitted to stand for 5 days, and in which there were no crystals by microscopic examination, showed only a faint purple color. Aerosol MA was found not to interfere with the reaction. According to Feigl, this test is specific for tyrosine in the presence of other amino acids. However, similar reactions are given by other *p*-substituted phenols and by proteins. Although the crystals have not been isolated in pure form, they are almost certainly tyrosine crystals, since they have the morphology and identical crystallographic properties of known tyrosine crystals and give a strongly positive test for tyrosine in material containing large numbers of the crystals.

DISCUSSION

This work shows that tyrosine crystals may be formed from leukocytes by the action of a synthetic detergent. Also, that there is no significant difference in the incidence of tyrosine crystal formation from leukocytes in patients with malignant disease, patients with destructive nonneoplastic diseases, and in normal persons. Although no significant difference was found in this group of persons, it is to be emphasized that these studies were on a qualitative basis, and that delicate tests for tyrosine exist that may show a significant difference. Wartman and Shlimovitz (*7*) have evolved a quantitative test for tyrosine on serum using the phenol reagent of Folin and Ciocalteu. Also, Lichtman and Sobotka (*8*) have developed a quantitative test in urine.

We have also shown that the tyrosine crystals are not derived from red cells or from plasma, since tyrosine crystals were not formed from these materials on exposure to the synthetic detergent and only developed from the buffy layer.

Of interest is the fact that the leukocyte is dopa-oxidase positive, that is, the cell contains an enzyme capable of conversion of dihydroxylphenylalanine, a derivative of tyrosine, into melanin.

There is a relationship between tyrosine metabolism and ascorbic acid. It has been shown by Butler and Cushman (*9*) that the buffy layer of the blood contains a reducing substance that chemically satisfies the criteria

used for ascorbic acid. They state that concentration of this reducing substance (ascorbic acid) in leukocytes in human beings and in guinea pigs depends on the supply of ascorbic acid in the diet, and provides a good index of significant deficiency of ascorbic acid. Sealock and Silberstein (*10*) found that in scorbutic guinea pigs there is a disturbance in tyrosine and phenylalanine metabolism manifested by excretion of homogentisic acid in the urine. This metabolic disturbance is benefited by the administration of ascorbic acid.

Another interesting finding was the formation of large numbers of tyrosine crystals from the leukocytes in a patient with chronic myelogenous leukemia. According to Hawk *et al.* (*11*), one of the diseases in which tyrosinuria is found is leukemia. This work suggests that the origin of tyrosine in the urine in leukemia may be from the leukocyte. One may also speculate that an agent, possibly surface active, is present to convert the protein of the leukocyte into the amino acid, tyrosine. Another disease in which tyrosinuria is commonly found is acute yellow atrophy of the liver. Here, two mechanisms for tyrosinuria are postulated: (1) that with impairment of liver function there is diminished deamination of amino acids, and (2) that the necrotic liver tissue serves as a source for tyrosine (*12*).

It was found that the synthetic detergent was not necessary in all instances to effect the formation of tyrosine from leukocytes. Thus, tyrosine crystals formed in control preparations without a synthetic detergent. The function of the synthetic detergent was to accelerate the reaction. In the experimental preparations tyrosine crystals formed on the third day and had reached 24 percent on the sixth day, while the control preparation showed no crystals on the sixth and showed 24 percent on the twelfth day. This finding suggests that Aerosol MA acts much like the proteolytic enzymes responsible for autolysis. In support of this view is the fact that tyrosine crystals will form in the buffy layer without the addition of Aerosol MA, providing the preparations are permitted to stand for a long time at room temperature—a factor favoring autolysis. That autolysis of tissue may cause the formation of other types of crystals, is supported by the recently reported work on Charcot-Leyden crystals (*2*). That is, if tissue containing eosinophils is permitted to autolyze for several days, Charcot-Leyden crystals are formed that may be demonstrated in tissue sections. The same result may be obtained after exposure of fresh tissue containing eosinophils to Aerosol MA, but in a much shorter time. Also, if tissue is mixed with Aerosol MA for 24 hours, then fixed, sectioned, and stained, changes similar to autolysis are found. These changes are loss of cellular detail, an eosinophilic cast to the tissue, pyknosis of the nuclei, and absence of cellular response (unpublished observations). Also, Bond (*13*) showed in 1887 that the addition of putrid serum to blood resulted in the formation of crystalline hemoglobin. The crystallization may be accomplished by the addition of a surface-active agent to blood.

The development of tyrosine crystals by means of a synthetic detergent in malignant tissue was not too unexpected, since tyrosine crystals have

been found in necrotic tumor tissue. Thus, Lichtman (*14*) states that in degenerating carcinoma of the lung, tyrosine may be found in the sputum. However, tyrosine crystals were also developed in nonneoplastic tissue as splenic tissue and leukocytes. The development of many other types of crystals in both neoplastic and nonneoplastic tissue was surprising and requires further study.

Some information on tyrosine metabolism in patients with malignant disease is available. Duboff and Hirshfeld (*15*) demonstrated a tyrosinase inhibiting factor in the blood of patients with malignant disease. However, Stadie *et al.* (*16*) were unable to confirm this finding. Tyrosinemia and tyrosinuria may be found in patients with advanced malignant disease; the tyrosine is believed to be derived from necrotic tumor tissue. Thus, Asperger (*17*) reported tyrosine and leucine in the urine of a patient with carcinoma of the lung with metastases to the liver.

The mechanism of action of the synthetic detergent in the formation of tyrosine from leukocytes is not known. It is known, however, that the synthetic detergents among other actions have the ability to hydrolyze proteins into simpler components [Hawk *et al.* (*18*)]. It may be hypothecated that Aerosol MA hydrolyzes some component of the leukocyte into tyrosine. Also, Allen and Bodine (*19*) have found that protyrosinase may be activated by the surface-active agents, Duponal and Aerosol, and also by chloroform, urethan, and urea, and by heat. Putnam (*20*) has excellently reviewed the interactions of proteins and synthetic detergents. Anson (*21*) showed that a variety of synthetic detergents denature proteins at the isoelectric point, and keep the denatured protein in solution. Under the conditions of our experiments, in which concentrated hemoglobin and concentrated synthetic detergent were mixed, crystals of hemoglobin formed (fig. 6). Crystals of hemoglobin were similarly obtained in the blood of the dog (fig. 7), guinea pig, rabbit, and mouse. These crystals had the appearance of oxyhemoglobin crystals as illustrated by Reichert and Brown (*22*).

We have shown that the addition of a synthetic detergent to tissues results in the formation of various types of crystals. The basis for this statement is the obtaining of (1) hemoglobin crystals, (2) the formation of Charcot-Leyden crystals, (3) the formation of tyrosine crystals from leukocytes, (4) the obtaining of diverse types of crystals from neoplastic and nonneoplastic tissue, (5) the formation of crystals from colloid of the thyroid gland, and (6) the work of Cohen (*23*) who obtained a different type of crystalline bushy stunt virus with a synthetic detergent.

CONCLUSIONS

The addition of dihexyl sodium sulfosuccinate to the buffy coat of blood results in the formation of tyrosine crystals.

There is no significant difference in the incidence of formation of tyrosine crystals by this method in patients with malignant disease, in patients with nonneoplastic diseases, and in normal persons.

The tyrosine crystals develop from the buffy coat (leukocytes) and not from the red blood cells or plasma.

The addition of dihexyl sodium sulfosuccinate to cancerous and noncancerous tissue results in the formation of tyrosine crystals and other crystals of unknown nature.

ACKNOWLEDGMENT

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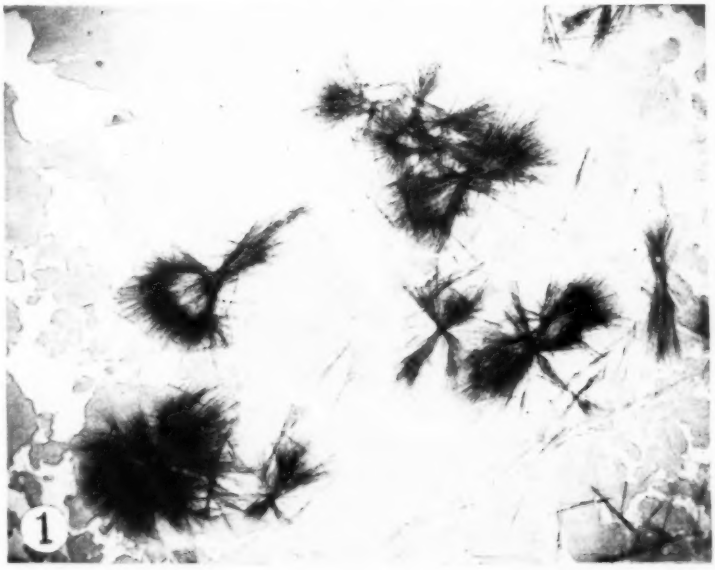
PLATE 183

FIGURE 1.—Tyrosine crystals in Aerosol MA-buffy-layer preparation from a patient with chronic myelogenous leukemia. ×100.

FIGURE 2.—Tyrosine crystal in Aerosol MA-buffy-layer preparation. ×400.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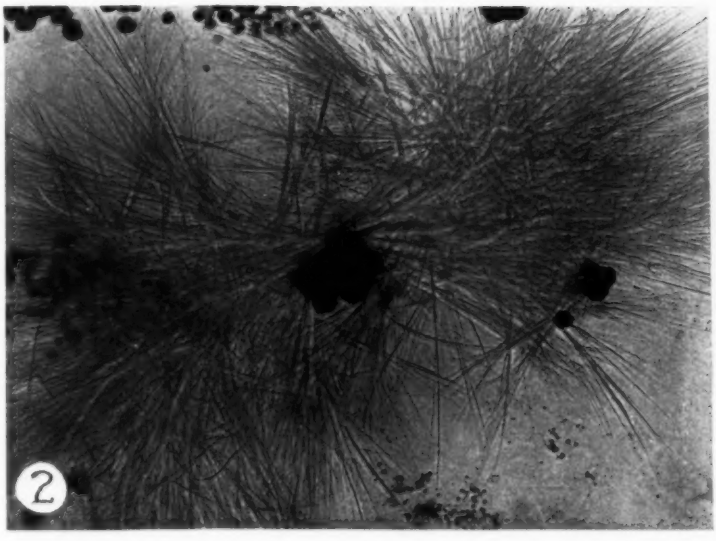
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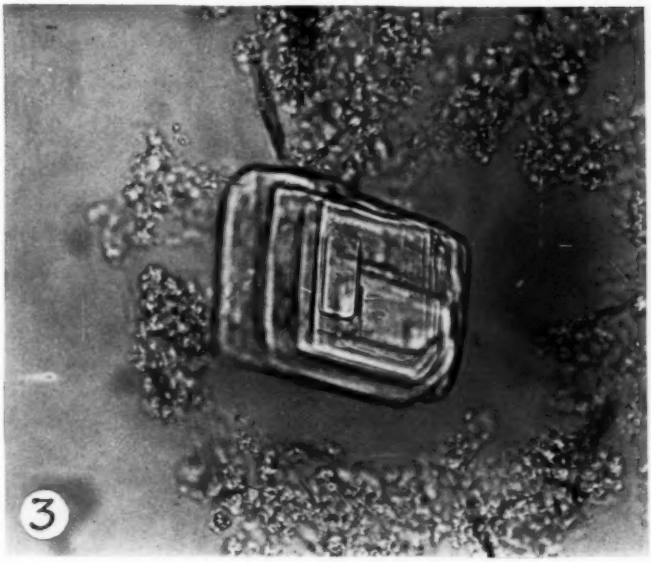
PLATE 184

FIGURE 3.—Crystal formed in Aerosol MA-buffy-layer preparation from a patient with chronic myelogenous leukemia; possibly phenylalanine. ×400.

FIGURE 4.—Crystal formed in cancer tissue—Aerosol MA preparation from a patient with adenocarcinoma of colon. ×400.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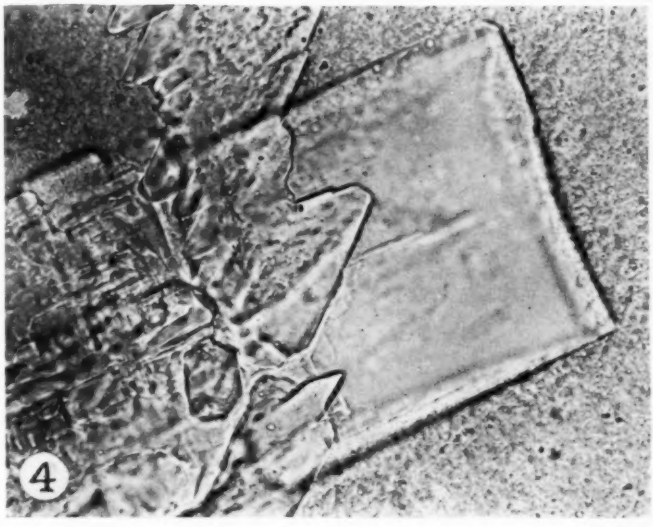
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PLATE 185

FIGURE 5.—Crystals formed in cancer tissue—Aerosol MA preparation from a patient with transitional-cell carcinoma of bladder. ×200.

FIGURE 6.—Hemoglobin crystals (human) formed in blood—Aerosol MA preparation. ×200.

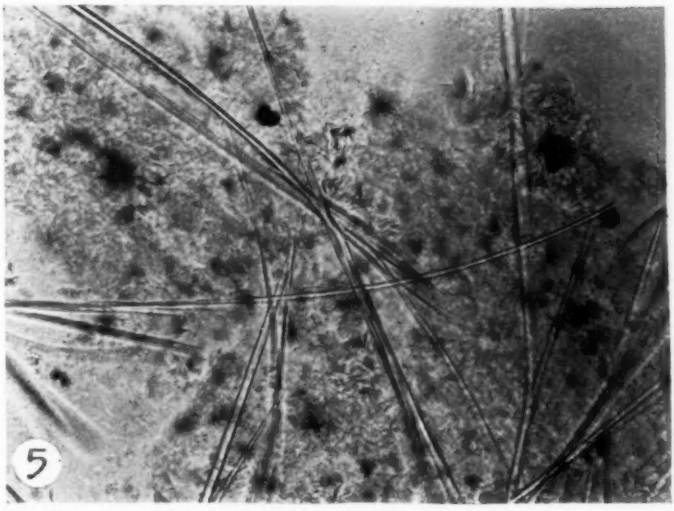
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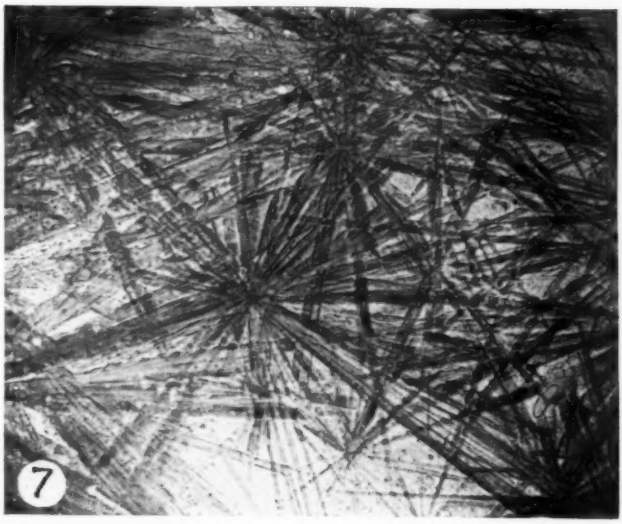
PLATE 186

FIGURE 7.—Hemoglobin crystals (dog) formed in blood—Aerosol MA preparation. ×200.

FIGURE 8.—Crystal formed in thyroid tissue—Aerosol MA preparations. ×400.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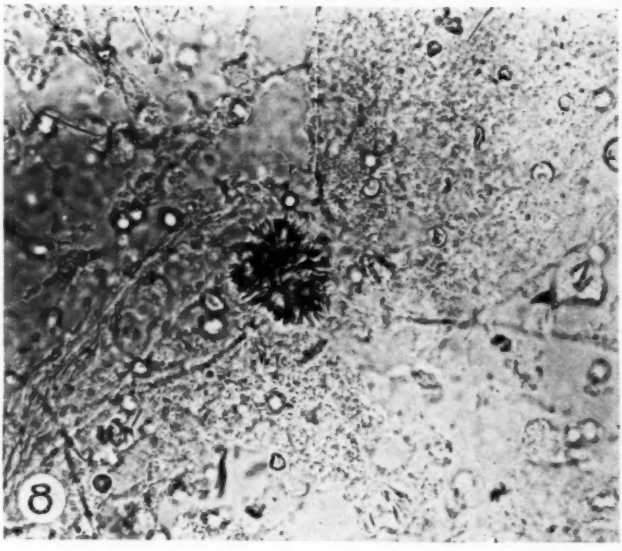
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NOTE ON THE INFLUENCE OF THE NUMBER OF LITTERS UPON THE INCIDENCE OF MAMMARY TUMORS IN MICE [1]

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It is agreed that in mice the incidence of mammary tumors in parous females is usually higher than in nonparous females of the same strain, and that if the incidence in parous and nonparous females is the same, the average tumor-age of parous females is earlier than that of virgins (*1–3*). Little attention, however, has hitherto been paid to the influence of the number of litters upon the incidence of mammary tumors. It seems doubtful whether an analysis of the breeding-behavior in relation to the incidence in a given strain would suffice to solve this question. To arrive at a conclusion on this subject it appears necessary to discontinue the breeding after a certain number of pregnancies. So far as can be ascertained from the literature, only Jones (*4*) has followed this method. In breeders of strain A, a direct quantitative relationship was found between the number of litters and the incidence of mammary tumors. The incidence increased, and the average tumor-age fell earlier, in proportion to the increase in the number of pregnancies. The question whether the number of litters is also of significance in the breeding method of Bagg (*5*), which is known as force-breeding, has apparently not yet been investigated. In the course of the present investigation the incidence of mammary tumors was determined in force-bred mice after two and after more than three pregnancies.

MATERIALS AND METHODS

The following strains and hybrids were used:

1. ♀ d=dilute-brown (dba) high-cancer-strain.
2. ♀ B=C57 black (Little) low-cancer-strain.
3. ♀ Bd=hybrids from mating B-females to d-males.
4. ♀ Bd=hybrids from mating B-females to d-males, immediately after birth, foster nursed on ♀ d throughout the entire lactation period.
5. ♀ 020=020 Leeuwenhoekhuis low-cancer-strain.
6. ♀ d0=hybrids from mating d-females to 020-males.

All mice were raised in the laboratory and fed commercial pellets and tap water *ad libitum*.

Virgins: After weaning, four or five animals were kept in a cage.

[1] Received for publication, March 10, 1950.

TABLE 1.—*Occurrence of mammary-gland tumors or deaths in virgin and force-bred mice with various numbers of litters*

Mice developing tumors / Mice dying without tumors

	Number	Average number of litters	Age in months													
			6m	7m	8m	9m	10m	11m	12m	13m	14m	15m	16m	17m	18m	19m	20m
♀ d																	
Virgins	86							1/2	1/1	4/2	3/2	6/2	6/6	6/2	2/3	4/3	1/2
Force bred 2x	48	2			1/0		0/1		2/0	2/1	4/1	1/1	3/0	2/2	6/0	5/0	3/3
Force bred >3x	69	7		1/0	3/0	3/0	6/0	11/0	4/0	8/0	14/0	2/0	4/1	4/0	3/0	3/1	
♀ Bd foster nursed by ♀ d																	
Virgins	70								1/0	2/0	1/0	3/0			4/1	9/1	5/1
Force bred 2x	58	2									3/1	1/0	1/2	6/1	6/2	5/4	4/2
Force bred >3x	56	11			2/0	2/0	4/1	1/0	6/0	5/0	8/0	2/0	2/0	3/0	6/0	4/0	1/1
♀ Bd controls																	
Virgins	73								0/1			0/1		0/2	0/1	0/5	0/3
Force bred >3x	61	13								0/1	0/1	0/1	0/2	0/2		1/1	0/3
♀ d0																	
Virgins	62		1/0		1/0	0/1	2/0	7/0	9/0	6/0	3/0	11/1	5/0	6/0	1/2	2/0	2/0
Force bred 2x	17	2			1/0		2/0	1/1	4/0	1/1	3/0	1/0	1/0	1/0			
Force bred >3x	29	9	1/0	4/0	4/0	2/0	2/0	1/0	1/0	2/0	2/0	4/0	1/0	1/0	1/0	2/0	1/0

Mice developing tumors / Mice dying without tumors

	Number	Average number of litters	Age in months										Total	Tumor incidence	Average age at which tumors occurred	Average age at death without tumor
			21m	22m	23m	24m	25m	26m	27m	28m	29m	30m				
														Percent	*Days*	*Days*
♀ d																
Virgins	86	------	7/4	2/0	3/1	0/4	4/1	------	1/0	------	------	------	51/35	59	546	537
Force bred 2x	48	2	0/3	1/3	0/2	0/1	------	------	------	------	------	------	30/18	62	477	586
Force bred >3x	69	7	------	------	1/0	------	------	------	------	------	------	------	67/2	97	382	533
♀ Bd foster nursed by ♀ d																
Virgins	70	------	5/2	6/1	4/2	2/4	0/4	0/1	3/3	1/1	0/2	0/1	46/24	66	608	729
Force bred 2x	58	2	1/2	5/3	0/2	3/0	0/1	0/2	0/1	------	------	------	35/23	60	565	616
Force bred >3x	56	11	0/1	1/0	------	2/0	0/1	0/1	0/1	0/1	------	------	49/7	88	441	682
♀ Bd controls																
Virgins	73	------	0/2	1/10	0/12	0/5	0/8	0/7	0/4	0/4	0/4	0/3	1/72	1	667	710
Force bred >3x	61	13	0/2	0/5	0/5	0/3	0/3	0/8	0/8	0/11	0/2	0/2	1/60	2	565	724
♀ d0																
Virgins	62	------	------	1/1	------	------	------	------	------	------	------	------	57/5	92	422	489
Force bred 2x	17	2	------	------	------	------	------	------	------	------	------	------	15/2	88	382	360
Force bred >3x	29	9	------	------	------	------	------	------	------	------	------	------	29/0	100	365	------

Force-breeding: Four females were kept with one male. The young born were removed as soon as possible after birth. At the end of the breeding period, four or five females were kept in a box. In the first group, force-breeding was stopped after two pregnancies; in the second group, the females were allowed to have as many litters as they would. All animals of the second group had more than three litters.

Observations were made at weekly intervals, tumors being noted as appearing on the date when first palpable. Histologic diagnoses were made in all cases. The animals listed in the table include only those that survived to tumor-age as determined by the age of the first animal to develop a tumor.

RESULTS

The results of the investigation are summarized in table 1. Females of the dilute-brown strain were used in the first experiment. No difference in the incidence of mammary tumors could be demonstrated between virgins and biparous females. However, the average tumor-age of parous females was more than 2 months earlier. Comparison of the group with an average of seven pregnancies with the biparous group showed that in the former the incidence of tumor was considerably higher and the average tumor-age 3 months earlier (382 days against 477 days). Similar results were obtained with hybrids of the d strain and the noncancerous strains C57 black and 020 Leeuwenhoekhuis. Biparous animals had mammary tumors at an earlier age than virgins, and showed an approximately equal incidence of tumors. With an increasing number of litters the incidence of tumors increased, and the tumor-age was considerably younger. It appeared from the control group ♀ Bd, without the mammary tumor agent, that force-breeding *per se* did not cause an increase in the incidence of tumors.

SUMMARY

In mice of the dilute-brown strain and their hybrids no difference in the incidence of mammary tumors could be demonstrated between virgins and biparous force-bred females. However, the average tumor-age of parous females was earlier. Force-bred females with more than three litters had a higher incidence of mammary tumors.

When determining the influence of pregnancy on the incidence of mammary tumors by comparison, the number of litters should be taken into account.

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LACTIC DEHYDROGENASE ACTIVITY OF CERTAIN TUMORS AND NORMAL TISSUES[1]

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The accumulation of lactic acid in many neoplasms may be due to a high rate of glycolysis or to decreased oxidation of this metabolite, or to both. Although tumor glycolysis has received considerable attention, few investigations have apparently been made of the relative activity in normal and neoplastic tissues of the system responsible for the reversible oxidation of lactate to pyruvate. Reports have appeared describing both the absence (*1*) and the presence (*2*) of lactic dehydrogenase in tumors. Dialyzed extracts of Jensen sarcoma and of rat muscle were found to exhibit about the same lactic dehydrogenase activity (*3*). On the other hand, Elliott *et al.* (*4*) observed that oxidation of lactate to pyruvate was deficient in slices of Philadelphia sarcoma No. 1 and Walker carcinoma. Lenta and Riehl (*5*) have recently reported that dialyzed suspensions of acetone powder preparations of transplanted mouse dbrB adenocarcinoma and sarcoma 180 possessed less lactic dehydrogenase activity than did similar preparations obtained from normal mouse tissues.[2] In order to characterize the enzymatic properties of neoplastic tissues, it is necessary to compare the relative activities of a number of tumors with their tissues of origin and with other normal tissues (*7*). In an attempt to approach such a characterization for lactic dehydrogenase activity, observations have been made on a variety of normal and neoplastic animal tissues employing an assay procedure that permits determination of activity in freshly prepared homogenates.

MATERIALS AND METHODS

The following neoplasms[3] were employed: (a) Transplanted hepatoma

[1] Received for publication, March 21, 1950.

[2] The observation that lactic dehydrogenase isolated from Jensen sarcoma was identical with that isolated from normal rat muscle on the basis of chemical, physical, enzymatic, and immunological criteria (*6*) supports the concept that enzymatic differences between normal and neoplastic tissues are quantitative rather than qualitative. (*7*).

[3] The author is indebted to the following members of the National Cancer Institute for generously providing the tumors used in this study: Dr. A. B. Eschenbrenner (a,n), Dr. C. D. Larsen (c), Dr. H. B. Andervont (b), Dr. E. Lorenz (d,e), Dr. A. J. Dalton (f), Dr. M. Belkin (g), Dr. H. L. Stewart (h), Dr. T. B. Dunn (i), Dr. M. W. Woods (j,k), Dr. B. Bennison (l), Dr. M. K. Barrett (m,r), Dr. H. I. Firminger (o), Dr. W. E. Heston (q), and Dr. J. White (r). The rhabdomyosarcomas were kindly donated by Dr. G. L. Miller of the Institute for Cancer Research, Philadelphia, Pa.

112 B, originally induced by chloroform (*8*); (b) transplanted hepatoma 13/8, originally induced by azotoluene (*cf. 9, 10*); (c) primary lung tumor, induced by urethan (*11*); (d) transplanted lymphoma No. 1, which arose originally in the mediastinum of a strain A mouse treated with X-radiation at birth (*12*); (e) transplanted lymphoma No. 2, which arose originally in the lymph nodes of a Strain A mouse treated with X-radiation at birth;[4] (f) transplanted thymoma, a tumor of the malignant lymphoma type, which arose originally in the thymus;[5] (g) transplanted sarcoma 37; (h) transplanted gastric adenocarcinoma, (*cf. 13*); (i) transplanted myoepithelioma, which arose originally in the submaxillary gland of a Strain C mouse (*14, 15*); (j) transplanted Harding-Passey melanoma (*16*); (k) transplanted melanoma S91 (*17*); (l) spontaneous mammary adenocarcinoma; (m) transplanted mammary adenocarcinoma (*18*); (n) transplanted granulosa-cell tumor (*cf. 19*), the histologic and endocrinologic aspects of which are similar to those described by Furth (*20*);[6] (o) induced interstitial cell tumor of testis (*21*); (p) transplanted rhabdomyosarcoma, originally induced with methylcholanthrene; (q) primary lung tumor, induced by methylcholanthrene; (r) primary rat hepatoma, induced by feeding p-dimethylaminoazobenzene; (s) transplanted fibrosarcoma AXCMCA2 originally induced with methylcholanthrene in a rat of the Dunning-Curtis strain (AXC9935).[7]

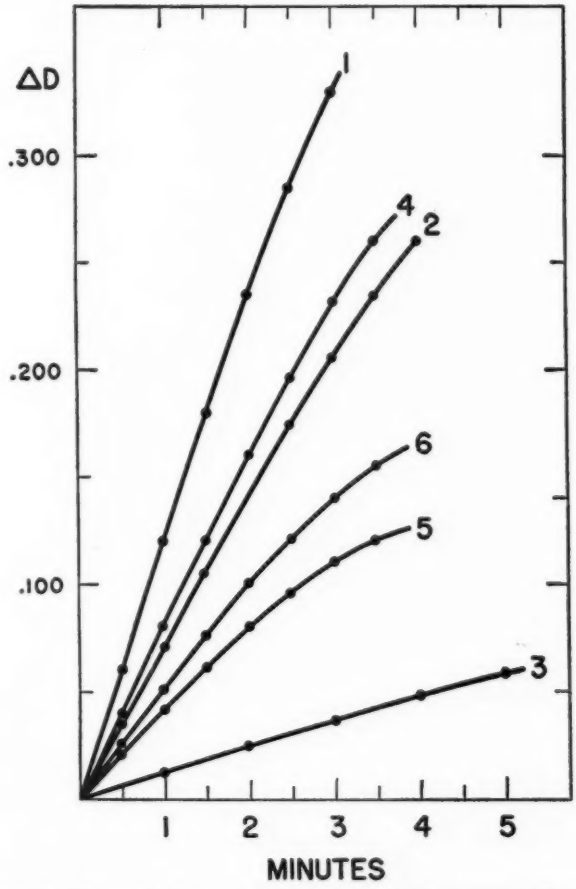
The animals were killed by cervical dislocation or decapitation followed by exsanguination, and the fresh tissues were homogenized with ten volumes of ice-cold distilled water in a glass Potter-Elvehjem homogenizer immersed in cracked ice. The homogenates were diluted with 10 to 40 volumes of distilled water immediately prior to the determination of activity. It has been reported that extracts of rabbit tissues ground with sand and distilled water exhibited no lactic dehydrogenase activity, but that grinding in the presence of salts (especially phosphate) resulted in active preparations (*22*). Under the present conditions, the values for homogenates prepared with distilled water and those prepared with 0.1 M phosphate buffer (pH 7.4) were identical for liver, skeletal muscle, and cardiac muscle of the rabbit and mouse, and for liver of the rat. Although it is possible that salts may in certain cases protect to an extent against enzyme inactivation, it is probable that destruction of enzyme is minimized when homogenization is performed rapidly and in the cold.

In determining lactic dehydrogenase activity in the direction of pyruvate formation, considerable quantities of cyanide have been added to fix pyruvate in order to obtain a linear time-activity relationship (*22*). Although this procedure has been used successfully in preventing inhibition by pyruvate formed in the course of the reaction in certain systems, in the present study of a number of different tissues, it seemed desirable to avoid this complication by determining the rate of the pyruvate→lactate reaction. Lactic dehydrogenase activity may be readily measured

[4] Lorenz, E., Personal communication.
[5] Dalton, A. J., Personal communication.
[6] Eschenbrenner, A. B., Personal communication.
[7] Barrett, M. K., Personal communication.

in the direction of lactate formation by a direct spectrophotometric method (*6*). The reaction proceeds more rapidly in this direction and goes to completion.

Enzyme activity was determined by measurement in a Beckman spectrophotometer of the rate of decrease in the absorption of dihydrodiphosphopyridine nucleotide ($DPNH_2$) at 3,400 A in a reaction mixture containing 0.25 micromole $DPNH_2$, 10 micromoles sodium pyruvate, 165 micromoles nicotinamide, 100 micromoles phosphate buffer (pH 7.4), and tissue homogenate in a volume of 3 cc. in a 1-cm. cell. Readings were made against a reference cell containing all components



TEXT-FIGURE 1.—Time-activity curves for lactic dehydrogenase activity of mouse tissues.

(1) skeletal muscle; (2) liver; (3) lung; (4) hepatoma 112B; (5) sarcoma 37; (6) urethan-induced lung tumor. The data were obtained with 0.1 cc. of a 1:400 homogenate under conditions described in the text. Ordinate=optical density change at 3,400 A.

except $DPNH_2$. The reaction was started by addition of 0.1 cc. of homogenate, and readings were taken at 30- or 60-second intervals for several minutes. All assays were carried out at 25–26° C. No $DPNH_2$ breakdown was observed with any of the tissues investigated in the absence of pyruvate under these conditions. The time-activity curves were linear over an appreciable range for homogenates of all tissues studied. Representative curves are presented in text-figure 1. Activity values were obtained from the initial portion of these curves and are expressed in terms of total homogenate nitrogen values determined by the Kjeldahl technique.[8] Enzyme activity was proportional to homogenate concentration under the conditions of this procedure, and increasing the concentrations of pyruvate and $DPNH_2$ to four times that stated above did not alter the rate of the reaction. The $DPNH_2$ was prepared (*23*) from DPN purified by counter-current distribution (*24*) and was 90 percent pure on the basis of Ohlmeyer's value for the extinction coefficient of reduced coenzyme (*23*). The keto acids employed were prepared and characterized as previously described (*25*, *26*) .

RESULTS

Investigation of the lactic dehydrogenase activity of mouse tissues revealed the presence of this enzyme in all normal and neoplastic tissues studied (table 1). The highest activity was found in skeletal muscle and intestinal mucosa, while pancreas, testis, spleen, uterus, thymus, lung, ovary, and submaxillary gland exhibited the lowest values. On the basis of the present data, differences in the activity of liver, kidney, and skeletal muscle of mice of strains A, C, and C3H are not apparent. Transplanted hepatoma 112B exhibited somewhat more activity than liver, and hepatoma 13/8 possessed about the same activity as liver. The primary lung tumors were five to seven times more active than lung, whereas the activity of the rhabdomyosarcoma was approximately four times lower than that of skeletal muscle. The activity values for thymoma, granulosa-cell tumor, gastric adenocarcinoma, lymphoma No. 2, and myoepithelioma were higher than those for the corresponding organs of origin. The values for the mammary tumors and the testicular tumor were about the same as those for lactating breast and testis, respectively. Such comparisons are obviously complicated by differences in the cellular composition of the tumors and the normal organs of origin. However, it may be generally concluded on the basis of these studies that the activity level of the tumors lies in approximately the middle of the range of the activity values for the normal tissues studied. In the case of the several rat tissues examined, adult liver showed somewhat less activity than fetal liver, and about the same activity as primary hepatoma and the transplanted fibrosarcoma.

[8] Acknowledgment is appreciatively made to Mr. Robert J. Koegel for performing the total homogenate nitrogen determinations.

TABLE 1.—*Lactic dehydrogenase activity of certain tumors, normal tissues, and tissues of tumor-bearing animals*

Tissue	Strain	Number of samples[1]	Activity[2]	
			Range	Mean value
MOUSE TISSUES				
Liver	A	4	361–490	420
Liver	C3H	4	390–480	435
Liver	C	4	364–474	429
Liver (mice bearing transplanted mammary tumor).	C3H	4	400–510	448
Liver (mice bearing transplanted hepatoma 112B).	A	4	415–523	465
Liver (mice bearing transplanted sarcoma 37).	C	5	390–500	455
Kidney	A	5	284–426	354
Kidney	C3H	4	360–400	380
Kidney	C	4	330–405	374
Kidney (mice bearing transplanted mammary tumor).	C3H	4	355–395	379
Kidney (mice bearing transplanted hepatoma 112B).	A	4	296–355	331
Kidney (mice bearing transplanted sarcoma 37).	C	5	350–405	377
Skeletal muscle	A	5	870–1, 030	948
Skeletal muscle	C3H	8	930–1, 020	979
Skeletal muscle	C	5	900–1, 040	974
Skeletal muscle (mice bearing transplanted mammary tumor).	C3H	4	940–1, 100	1, 010
Skeletal muscle (mice bearing transplanted hepatoma 112B).	A	4	970–1, 050	1, 000
Skeletal muscle (mice bearing transplanted sarcoma 37).	C	5	975–1, 020	995
Cardiac muscle	C3H	4	276–312	292
Lung	A	5	77. 6–93. 1	82. 5
Intestine	C3H	3	445, 450, 515	470
Intestinal mucosa	C3H	4	676–770	730
Stomach	C3H	3	231, 253, 300	261
Gastric mucosa	C3H	3	179, 201, 223	201
Pancreas	C3H	3	135, 162, 168	155
Spleen	C3H	4	125–154	140
Submaxillary gland	C	4	112–156	136
Brain	C3H	4	210–232	226
Thymus[3]	C3H	4	186–192	189
Lymph nodes	C3H	3	201, 203, 215	206
Uterus	C3H	3	134, 138, 149	140
Ovary	C3H	3	100, 123, 125	116
Lactating breast	C3H	3	324, 372, 423	373
Testis	C	5	167–228	188
Transplanted hepatoma 112B	A	7	594–765	669
Transplanted hepatoma 13/8	C	4	334–441	381
Primary lung tumor (urethan-induced)	A	6	430–520	466
Primary lung tumor (methylcholanthrene-induced).	CAF1	5	480–642	561
Transplanted lymphoma No. 1	CAF1	3	292, 312, 273	292
Transplanted lymphoma No. 2	A	6	293–362	335
Transplanted thymoma	DBA	3	286, 314, 348	316
Transplanted sarcoma 37	C	6	374–499	419
Transplanted gastric adenocarcinoma	C3H	4	302–360	336
Transplanted Harding-Passey melanoma	C	4	133–188	163
Transplanted melanoma S91	DBA	3	326, 383, 393	367
Spontaneous mammary adenocarcinoma	C3H	5	246–342	299
Transplanted mammary adenocarcinoma	C3H	4	315–440	389
Transplanted granulosa-cell tumor	C3H	3	245, 251, 276	257
Induced interstitial-cell tumor of testis	C	3	172, 194, 205	190
Transplanted myoepithelioma	C	5	409–565	492
Transplanted rhabdomyosarcoma	C3H	5	208–284	250

Footnotes at end of table.

TABLE 1.—*Lactic dehydrogenase activity of certain tumors, normal tissues, and tissues of tumor-bearing animals*—Continued

Tissue	Strain	Number of samples[1]	Activity[2]	
			Range	Mean value
RAT TISSUES				
Adult liver	O–M[4]	5	367–471	419
Fetal liver[5]	O–M	4	599–679	640
Primary hepatoma	O–M	3	476, 516, 494	495
Placenta[5]	O–M	4	114–149	127
Transplanted fibrosarcoma AXCMCA2	D–C[6]	3	390, 444, 409	414

[1] Each sample represents tissue from a single animal or pooled tissues of several animals.
[2] Activity is expressed as moles $\times 10^{-9}$ of pyruvate reduced per milligram of total homogenate nitrogen per minute.
[3] Animals 6–9 weeks of age.
[4] Osborne-Mendel.
[5] 12th–14th days of gestation.
[6] Dunning-Curtis.

The activity of the liver, kidney, and skeletal muscle of mice bearing transplanted hepatoma 112B, transplanted sarcoma 37, and transplanted mammary adenocarcinoma was not significantly different from the respective normal levels. In these experiments only animals bearing tumors weighing 5 percent or more of the body weight were employed. Although the lactic dehydrogenase activity of these tissues was within the normal range, considerable reduction (50 percent or greater) was observed in the liver catalase activity of animals bearing transplanted sarcoma 37 and transplanted mammary adenocarcinoma[9] (*cf.* 7). The absence of a systemic effect of the tumor on lactic dehydrogenase contrasts markedly with that on catalase, and is in agreement with observations on dbrB adenocarcinoma and sarcoma 180 (*5*).

In previous studies it was found that crystalline beef heart lactic dehydrogenase and preparations of rabbit skeletal muscle, pigeon breast muscle, and hog heart reduced α-keto and α, γ-diketo acids at similar relative rates in the presence of $DPNH_2$. Electrophoretic and enzymatic evidence supported the possibility that a single enzyme was responsible for reduction of both classes of substrates (*25*). It therefore seemed of interest to determine the rates of reduction of other keto acids by tumors. The

TABLE 2.—*Reduction of α-keto and α, γ-diketo acids*[1]

	Pyruvic acid (a)	α-keto butyric acid (b)	α, γ-diketo-valeric acid (c)	Ratio (a) : (b) : (c)
Normal mouse skeletal muscle	990	578	103	1,000 : 584 : 104
Transplanted rhabdomyosarcoma	273	160	26. 0	1,000 : 586 : 95
Transplanted gastric adenocarcinoma	347	228	36. 5	1,000 : 656 : 105
Spontaneous mammary adenocarcinoma	369	231	38. 2	1,000 : 626 : 104
Crystalline lactic dehydrogenase[2]	167, 000	95, 600	19, 800	1,000 : 573 : 119

[1] Values expressed as in table 1; substrate concentration: 5 micromoles per 3 cc.
[2] Prepared from beef heart (*27*).
[3] Greenfield, R. E. and Meister, A., Unpublished data.

data obtained on the reduction of pyruvic, α-ketobutyric, and α, γ-diketovaleric acids by homogenates of normal mouse skeletal muscle, rhabdomyosarcoma, gastric adenocarcinoma, spontaneous mammary adenocarcinoma, and crystalline lactic dehydrogenase are given in table 2. The activity ratios for the reduction of these three keto acids by the several tissue preparations were about the same, thus providing additional evidence compatible with the single enzyme hypothesis.

DISCUSSION

Lactic dehydrogenase activity cannot be correlated with the values reported for the glycolytic rates of various tissues (*28*). In the present study the activity of the apodehydrogenase has been specifically measured, and the values obtained probably represent the maximum activity of the tissues. Deficient oxidation of lactate to pyruvate in tumors could result from a low concentration of coenzyme (*29*, *30*, *31*) or an excess of reduced coenzyme. Although the ratio of $DPNH_2$ to DPN was found to be much higher in Jensen sarcoma than in a variety of normal tissues (*2*), further studies have revealed that this phenomenon is not a general characteristic of all tumors (*32*).

The tumors studied exhibited about the same lactic dehydrogenase activity, or a higher one, than the corresponding tissues of origin, except in the case of rhabdomyosarcoma. Although the activity of rhabdomyosarcoma was much less than that of normal skeletal muscle, and the values for lung tumors were considerably greater than those for normal lung, the mean values for these tumors (250, 466, and 561) were relatively close compared to those for the tissues of origin (979 and 83). The extreme range of values for the mouse tumors was 163 (Harding-Passey melanoma) to 669 (hepatoma 112B), or about one-third of that for the normal tissues (83 for lung, to 979 for muscle). The data thus provide another example of the relatively uniform enzymatic behavior of tumors (*7*). On the basis of the present findings it would appear that there is no appreciable deficiency of lactic dehydrogenase in tumor tissues.

SUMMARY

The lactic dehydrogenase activity of 19 tumors and 20 normal tissues of the mouse and rat was investigated employing a spectrophotometric assay procedure based upon the rate of pyruvate reduction, and followed by observation of the change in absorption of dihydrodiphosphopyridine nucleotide. The values for the tumors studied were higher than, or the same as, the corresponding tissues of origin except in one case. The greatest differences were observed between normal mouse skeletal muscle and rhabdomyosarcoma, and between lung and lung tumors. The variation in activity among the tumors was about one-third that of the normal tissues. It was concluded that tumors possess appreciable lactic dehydrogenase activity, and that the level of activity in tumors lies in about the middle of the range for normal tissues. Tumors reduced pyruvic, α-ketobutyric, and α, γ-diketovaleric acids in the presence of

dihydrodiphosphopyridine nucleotide at approximately the same relative rates as did normal mouse skeletal muscle and crystalline beef heart lactic dehydrogenase.

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DAMAGE INDUCED IN SARCOMA 37 WITH PODOPHYLLIN, PODOPHYLLOTOXIN ALPHA-PELTATIN, BETA-PELTATIN, AND QUERCETIN [2]

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INTRODUCTION

In 1942 Kaplan (*6*) reported the use of podophyllin in the topical treatment of condylomata acuminata. King and Sullivan (*7*) in 1946 described the colchicine-like effect of this drug on cells of normal rabbit and human skin. The following year podophyllin was reported by Belkin (*8*) and by us (*1*), to be capable of affecting transplanted tumors in mice following subcutaneous injection of the drug. Ormsbee and Cornman (*9*) stated that it damaged tumor cells *in vitro*. The extensive necrosis induced in tumors with podophyllin in the *in vivo* experiments led us to undertake the isolation of the active agent, or agents, from the crude drug.

In this laboratory, fractionation yielded two crystalline compounds with high potency in producing damage to Sarcoma 37. In a report presented by Hartwell and Shear in May 1947, one of these potent substances was identified as podophyllotoxin; the second, a compound (No. 1074) hitherto undescribed, was later named *alpha-peltatin* by Hartwell (*10*). Still another new crystalline compound of high potency was later isolated from podophyllin; this substance, described in a report by Hartwell and Detty (*11*), was named *beta-peltatin*.

The mandrake, or May apple, root has been in use for a long time as a purgative. At present it is an article of commerce, where it is said to find its way into proprietary medicines. The dried rhizomes and roots of the American plant, *Podophyllum peltatum L.*, as well as its alcohol-soluble resin (*podophyllin*) have been used in pharmacy, and were listed in various pharmacopoeias, dispensatories, and formularies. Another variety (*P. emodi Wall.*) is obtained from India. In 1898 Dunstan and Henry (*12*), insofar as their work went, found the same components present in *P. peltatum* and *P. emodi*. The work reported here was restricted to the American variety.

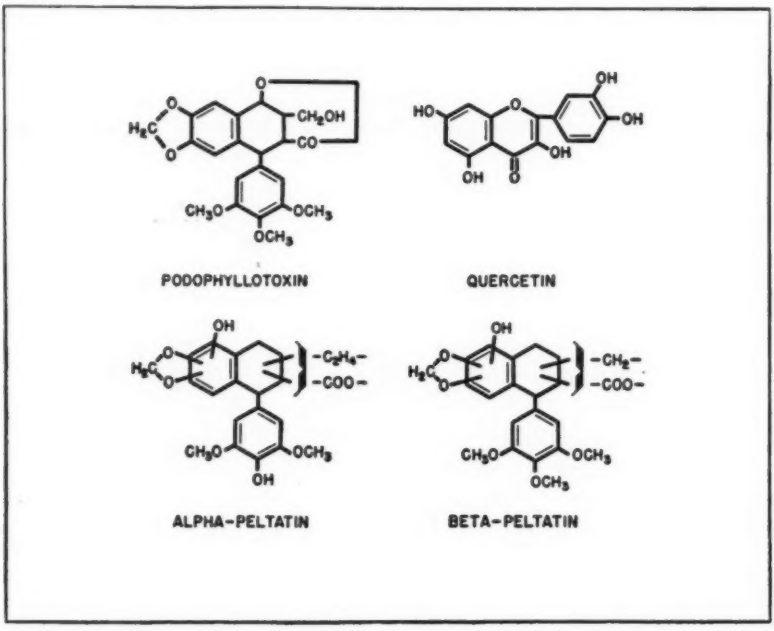
Podophyllin has been reported to contain many substances, but later

[1] Received for publication, April 11, 1950.

[2] Preliminary reports from this laboratory on these agents were presented, in part, at the Annual Meetings of the American Association for Cancer Research (*1–4*) in 1947, 1948, and 1949, and at the Fourth International Cancer Research Congress (*5*) in 1947.

work showed some of these statements to be in error. Only two of the substances previously isolated from the crude drug are well-defined crystalline substances, *viz.*, podophyllotoxin ($C_{22}H_{22}O_8$) and quercetin ($C_{15}H_{10}O_7$). Picropodophyllin, an isomer of podophyllotoxin, was described in 1881 by Podwyssotzki (*13*) as having been isolated from podophyllin. It is clear from subsequent work (*12, 14, 15*) that picropodophyllin is an alkali conversion product of podophyllotoxin, and has not been obtained from podophyllin in the absence of alkali. Alpha-peltatin and beta-peltatin, the two new compounds recently isolated, are both isomeric with podophyllotoxin.

Podophyllotoxin was isolated by Podwyssotzki (*13*) in 1881; its currently accepted structure (see below) was proposed independently in 1932 by Borsche (*14*) and by Spaeth (*15*). Earlier workers (*12*) had reported the podophyllotoxin content of podophyllin to be about 20 percent, but the method of analysis they employed makes it probable that the peltatins were included in their data for the toxin. The figure of 9.6 percent reported by Hartwell and Detty (*16*) is probably more accurate.



Quercetin was isolated by Podywssotzki (*13*) who called it podophylloquercetin. However, it was later purified by Dunstan and Henry (*12*) who demonstrated it (text-fig. 1) to be identical with the pigment from quercitron bark.

The chemical properties of alpha- and beta-peltatin have been described by Hartwell and Detty (*16*) in a recent paper. They found about 5

percent of each of these two compounds in specimens of podophyllin studied. Text-figure 1 gives the tentative structures assigned to them.

This paper describes the effects on mouse Sarcoma 37 of the crude drug and of the compounds that have been isolated from it.

MATERIALS AND METHODS

The podophyllin used in these experiments was the N. F. grade purchased from two chemical companies (Penick and Baker). The podophyllotoxin, α-peltatin, and β-peltatin were isolated by Hartwell and Detty from these specimens; quercetin was prepared in this laboratory by the acid hydrolysis of rutin (*17*). The various preparations of these compounds had the following melting point ranges:

Compound:	*Melting point °C (corr.)*
podophyllotoxin	115–117
α-peltatin	230–232
β-peltatin	231–238
quercetin	308–313

For the preliminary toxicity studies, hybrid mice of various types were used. The mice bearing Sarcoma 37 were exclusively CAF_1. These first generation hybrids of female Strain C and male Strain A mice were chosen because the hybrids are uniform genetically, the litters are large, and the offspring sturdy. Sarcoma 37 grows progressively in this hybrid strain with a negligible number of spontaneous regressions (*18*).

Transplantation of Sarcoma 37 was carried out by injecting into the right thigh muscle approximately 0.05 cc. of a tumor mash diluted with about one volume of 0.9 percent NaCl solution. About 150 to 250 mice were implanted with tumor at the same time. For this number of animals, 8 to 12 tumors about 1 week old were pooled. The preparation of the tumor mash was carried out with aseptic precautions. Each tumor mash was tested for bacterial contamination by incubating an aliquot in nutrient broth and/or in a medium containing thioglycollate. When the broth showed contamination, the experiments were considered unreliable and were repeated with tumor mash yielding negative results for contamination. The infected tumors varied in their appearance from small, pus-containing growths to tumors grossly indistinguishable from satisfactory ones. In testing for sterility, tumor fragments were incubated prior to mincing; in addition, after completion of the implantation, an aliquot of the remaining tumor mash was also incubated in the broth.

The mice bearing Sarcoma 37 were employed when the tumors were 6 days old. At this time, they were usually between 0.5 and 1.0 cm. in diameter, were well vascularized, and showed little necrosis except for the original implant. Approximately 10 percent of the inoculated mice of each batch were set aside to serve as untreated controls; a group of control animals was sacrificed, for comparison, each time that treated mice from the same batch were killed (by cervical dislocation). More than 3,000 tumor-bearing animals were employed in the experiments described in this paper.

The chemical agents were injected subcutaneously in the left flank, contralaterally to the tumor implant, in mice bearing 6-day-old tumors. Aqueous solutions were used where feasible; in the case of water-insoluble compounds, a solution or suspension in olive oil was used. The concentrations were so chosen that the dose desired for injection was usually contained in 0.01 cc. for each gram of body weight.

At various times during the first 48 hours after injection (usually after 8, 24, and 48 hours), about five animals at a time were killed for each experiment, and the tumors examined for gross effects. Representative slices of the tumors were fixed in Zenker-formol, sectioned, stained with hematoxylin-eosin, and evaluated histologically.[3]

When tumor damage was induced, it was usually evident grossly from the extensive hemorrhagic areas in the tumors on comparison with the controls (fig. 1). Small areas of hemorrhage were not considered significant, inasmuch as this was occasionally seen in untreated Sarcoma 37. Tumors with induced damage frequently showed some softening. In general, tumor damage seen grossly was corroborated in the subsequent miscrosopic evaluation, particularly when widespread necrosis had been induced. The results summarized in the tables are the findings of the gross observations. In some instances, however, damage was seen microscopically although the gross observations were negative; these instances are indicated by an asterisk in table 4. In some of these instances, miscroscopic evidence of tumor damage shortly after treatment was followed by gross tumor damage in a few hours; in other instances, the damage was apparently reversible, inasmuch as no damage was seen the next day either grossly or microscopically.

The data reported here are for mice that were sacrificed; those that were found dead were discarded.

EXPERIMENTAL OBSERVATIONS

Effect of Components of Podophyllin

(A) *Injected in Oil Vehicle.* Suspensions or solutions of podophyllin, of podophyllotoxin, of alpha-peltatin, of beta-peltatin, and of quercetin were prepared by grinding each substance with olive oil. In concentrations above 0.1 percent only the podophyllotoxin formed a clear solution. A single subcutaneous injection of each drug was given to tumor-bearing mice at various dose levels; animals were sacrificed after 8, 24, and 48 hours and the tumors examined for induced damage. Table 1 illustrates the results obtained. Crude podophyllin consistently produced damage in Sarcoma 37 at doses below 40 micrograms per gram, the maximum tolerated dose (table 3). At 10 micrograms per gram, damage was induced in the tumors of only about half of the animals. Podophyllotoxin, alpha-peltatin, and beta-peltatin produced damage at dose levels down to 5 micrograms per gram. Below this dose, the results were variable; repeated experiments, with relatively large numbers of mice, are necessary

[3] By Drs. MacCardle and Downing (*8*). Complete reports of the histologic and cytologic criteria and findings are in preparation (*19*).

for the definitive determination of the "end-point" (*20*), *i. e.*, the dose which induces an effect in about 50 percent of the tumors. In sharp contrast, quercetin in olive oil suspension induced no perceptible damage in the tumors in doses up to 1,000 micrograms per gram. Since an aqueous vehicle was then found preferable, further work at the minimum dose level with these compounds was usually carried out with aqueous solutions.

TABLE 1.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of podophyllin and of its components, in oil*

Substance	Dose [1]	Number of experiments	Mortality [2]	Induced tumor damage [3] at:		
				8 hours	24 hours	48 hours
	μg./gm.					
Podophyllin	50	5	10/51	10/10	22/23	8/8
	30	2	0/14	5/5	9/9	--------
	10	2	1/13	--------	7/12	--------
Podophyllotoxin	18	1	0/8	--------	8/8	--------
	12	1	0/8	--------	8/8	--------
	6	3	0/24	--------	24/24	--------
	5	1	0/24	8/8	8/8	8/8
	3	4	0/34	--------	15/34	--------
	1	1	0/8	--------	0/8	--------
Alpha-peltatin	12	1	0/4	--------	4/4	--------
	6	1	0/6	--------	6/6	--------
	3	8	1/57	--------	38/56	--------
Beta-peltatin	20	1	0/5	--------	5/5	--------
	5	1	0/5	--------	3/5	--------
	3	1	0/5	--------	0/5	--------
Quercetin	1,000	1	0/15	0/5	0/5	0/5
	250	1	0/5	--------	0/5	--------

[1] Dissolved or suspended in olive oil; volume injected was 0.01–0.02 cc. per gm. of body weight.
[2] Expressed as: $\frac{\text{Mice dead}}{\text{Mice injected}}$.
[3] Expressed as: $\frac{\text{Number of mice with induced tumor damage}}{\text{Number of mice sacrificed}}$. The tumors in untreated controls showed no induced damage (see p. 1275).

The damage induced in the tumors after subcutaneous injection of a single large dose of the crude drug or of each of its active components followed a similar pattern, as seen grossly. Within 8 hours the tumors became somewhat softer as well as diffusely hemorrhagic. In 24 hours the tumors were completely hemorrhagic except for occasional islands, in some tumors, of apparently unaffected tissue. By 48 hours, the hemorrhage in the affected areas had begun to disappear; the previously hemorrhagic tumor tissue now consisted of discolored caseous necrotic material. At this time it was not unusual to observe regenerating tumor tissue at the periphery of the necrotic tumor. Complete destruction of the tumors was not observed. Figure 1 shows typical gross findings.

The microscopic observations paralleled the gross findings. Briefly, the earliest damage was observed (*19*) in about 2 hours and was characterized by a shrinkage of nuclei, retraction of cytoplasm, and marked eosino-

philia; these changes were termed "early cell damage." By 6 or 8 hours further degeneration occurred, and nuclei became markedly pyknotic with further cytoplasmic retraction and deep eosinophilia. Stasis was frequently observed. These later stages were termed "induced generalized pyknosis" or "induced necrosis." As in the gross observations, after 48 hours proliferating cells were frequently seen at the periphery of the tumor, while cellular disintegration was observed in the severely affected portion. Arrested or bizarre and distorted mitotic figures, or giant cells, were observed in the regenerating tissues at this time, indicating a possible residual action of the drug. Figure 2 illustrates some of the features which we have selected from the material of experiments reported here.

(B) *Injected in Aqueous Vehicle.* Since podophyllin is not soluble in water (the crude drug is prepared by precipitating an alcoholic extract of the dried, powdered roots and rhizomes with a large excess of water), the initial experiments with the drug and with the fractions obtained from it were carried out in olive oil suspension or solution. After the active compounds had been isolated and their chemical properties ascertained, the suitability of aqueous vehicles was investigated.

It has been reported (*21*) that the solubility of crude podophyllotoxin in water is 0.07 percent. In this laboratory [4] the solubility of the purified toxin was found (*16*) to be only 0.01 percent. Podophyllotoxin is quite soluble in propylene glycol; a 50 percent aqueous solution holds as much as 0.5 percent in solution. Both water and 50 percent aqueous propylene glycol were employed as vehicles.

Alpha-peltatin and beta-peltatin, as well as quercetin, have at least one phenolic hydroxyl group, and are therefore soluble in aqueous sodium hydroxide. In preparing dilute solutions of the peltatins, 0.05 to 0.1 cc. of normal alkali was sufficient to dissolve 10 mg. of compound. The final concentration of alkali required for 0.1 percent solutions of the peltatins was less than 0.01 N.

Table 2 illustrates the results obtained with aqueous vehicle. The toxin and both peltatins showed high tumor-damaging activity in doses down to about 2 micrograms per gram of body weight; damage was induced in some of the tumors with even less than 2 micrograms per gram in the case of podophyllotoxin and of the beta-compound. At low dose levels, therefore, the activity in water appeared to be somewhat greater than in oil. In agreement with the results obtained in oily vehicle, quercetin was inactive at doses up to 200 micrograms per gram. At higher doses, however, alkaline solutions of quercetin consistently produced damage in the tumors. Higher concentrations of alkali (0.2–0.3 N) were required to effect complete solution of the quercetin; extensive ulceration occurred at the site of the injection. Subcutaneous injection of sodium hydroxide in these concentrations alone produced no damage in the tumors although here, too, severe ulceration was produced at the injection site.

[4] In making a saturated aqueous solution of the toxin, care should be taken not to heat the solution above 60° C.; there is indication that conversion, possibly to picropodophyllin, may occur under these conditions.

TABLE 2.—*Damage induced in Sarcoma 37 with a single subcutaneous injection of aqueous solutions of podophyllin components*

Substance	Dose [a]	Number of experiments	Mortality [b]	Induced Tumor Damage [c] at:		
				8 hours	24 hours	48 hours
	μg./gm.					
Podophyllotoxin	[d] 20	2	0/24	8/8	8/8	8/8
	[d] 5	2	0/23	8/8	7/7	8/8
	[e] 4. 4	1	0/8	----	8/8	----
	[e] 3. 3	1	0/5	----	5/5	----
	[e] 2. 2	1	0/7	----	7/7	----
	[e] 1. 1	2	0/15	----	3/15	----
Alpha-peltatin [f]	160	2	11/13	----	2/2	----
	80	4	10/29	----	19/19	----
	40	1	1/8	----	7/7	----
	20	3	0/25	4/4	16/16	5/5
	5	7	0/68	14/14	40/40	14/14
	3	2	1/16	----	15/15	----
	2	4	0/32	----	15/32	----
	1	3	0/24	0/4	0/20	----
Beta-peltatin [f]	20	3	0/25	4/4	13/13	8/8
	5	2	0/29	8/8	13/13	8/8
	3	1	0/8	----	8/8	----
	2	1	0/8	----	8/8	----
	1. 5	2	0/16	----	8/16	----
	1. 0	3	0/20	4/4	0/12	0/4
Quercetin [f]	750	1	2/8	----	6/6	----
	500	2	0/20	4/4	12/12	3/4
	250	4	2/28	----	9/26	----
	200	3	1/28	3/4	6/19	2/4

[a] Dissolved in aqueous vehicle; volume injected was 0.01–0.04 cc. per gm. of body weight

[b] Expressed as: $\frac{\text{Mice dead}}{\text{Mice injected}}$.

[c] Expressed as: $\frac{\text{Number of mice with induced tumor damage}}{\text{Number of mice sacrificed}}$. The tumors in untreated controls showed no induced damage (see p. 1275).

[d] In 50 percent aqueous solution of propylene glycol.

[e] In saturated aqueous solution.

[f] In dilute aqueous NaOH.

MINIMUM EFFECTIVE DOSE AND TIME

After investigating, in acute experiments, the tumor-damaging action of the drugs during the first 48 hours, mice bearing 6-day-old implants of Sarcoma 37 were given, subcutaneously, a large dose of each of the drugs; survival of the animals was then noted over a period of 5 days. The largest dose in a single injection that caused no deaths (or, at most, deaths in only a small percent of the mice) was considered the Maximum Tolerated Dose (MTD). Table 3 summarizes the results obtained. The smallest dose that produced damage promptly in an appreciable percentage of the mice was considered the Minimum Effective Dose (MED). Both the MTD and MED showed some variation [5] for each of the compounds;

[5] In other experiments with these and other compounds, in non-tumor-bearing mice as well as in mice bearing other types of tumors, it was evident that the variations in the values were much greater in mice with tumors than in normal mice. Furthermore, the values for MTD and MED were somewhat different for the same compound in mice bearing different types of tumors. Finally, with a given type of tumor, the value was influenced by the age and the size of the tumor. Hence these numerical values are to be regarded not as definitive "constantsd but rather as empirical guides for a particular set of experimental conditions. These values for MTD an" MED should not be considered as characteristic of the chemical compound, but merely as a summary of the total biological situation under consideration.

the values given in table 3 represent an over-all summary of the data obtained. Apart from quercetin, which had a lower order both of activity and of toxicity, the three highly potent compounds each had an MTD about the same as that of the crude drug. For these last three compounds the MED in each case was, however, lower than that of podophyllin itself; the ratio MTD/MED ("therapeutic index") was, accordingly, greater than that for the crude drug.

TABLE 3.—*Approximate values for maximum tolerated dose (MTD) and minimum effective dose (MED) for single subcutaneous injection of podophyllotoxin, alpha-peltatin, beta-peltatin, and quercetin in mice bearing Sarcoma 37*

Substance	MTD	MED	$\frac{MTD}{MED}$
	μg./gm.	μg./gm.	
Podophyllin	40	10	4
Podophyllotoxin	30	2	15
Alpha-peltatin	40	2	20
Beta-peltatin	40	2	20
Quercetin	600	200	3

These four potent compounds were next injected into animals bearing Sarcoma 37 which were sacrificed 2, 4, 6, 24, and 48 hours after injection; the tumors were then examined for evidence of damage. This provided information on the minimum time after a single subcutaneous injection required for production of perceptible damage to the tumors. The results are summarized in table 4. With a large dose the gross changes were

TABLE 4.—*Minimum time required for inducing perceptible damage in Sarcoma 37 with a single subcutaneous injection of podophyllotoxin, alpha-peltatin, beta-peltatin and quercetin*

Compound	Dose	Number of mice	Induced tumor damage [1] at:				
			2 hours	4 hours	6 hours	24 hours	48 hours
	μg./gm.						
Podophyllotoxin [2]	20	20	4*/4	4*/4	4/4	4/4	4/4
	20	16	4*/4	4*/4	4/4	4/4	------
	5	20	4*/4	4*/4	4/4	4/4	4/4
	5	19	4*/4	4*/4	4*/4	3/3	3/4
	1	12	4*/4	2*/4	------	2*/4	------
Alpha-peltatin [3]	20	16	4*/4	4*/4	4/4	4/4	------
	20	20	5*/5	5*/5	------	5/5	5/5
	5	20	4*/4	4*/4	4*/4	4/4	4/4
	1	16	0/4	4*/4	0/4	0/4	------
Beta-peltatin [3]	20	16	4*/4	3*/4	------	4/4	4/4
Quercetin [2]	500	16	2*/4	------	4/4	3/4	4/4
	200	16	4*/4	------	3/4	4/4	2/4

[1] Expressed as: $\frac{\text{Number of mice with induced tumor damage}}{\text{Number of mice sacrificed}}$. The tumors in untreated controls showed no induced damage (see page 1275).
[2] In 50 percent aqueous solution of propylene glycol.
[3] In dilute aqueous NaOH.
*Represents "early cell damage" seen microscopically (*19*), but no grossly visible tumor damage.

noted in about 6 hours. Microscopically, evidence of "early cell damage" was observed[3] as early as 2 hours after injection. In the case of the toxin and of the alpha compound, a subeffective dose was examined in the same way; the subeffective dose (1 microgram per gram) produced no grossly visible changes in the tumor but occasionally showed microscopic evidence of "early cell damage." Moreover, this mild damage appeared to be reversible, inasmuch as many of the mice sacrificed later were completely negative, even microscopically. At higher doses, on the other hand, the damage became progressively more severe with time. In this set of experiments the tumor tissue, except for the slices fixed for histologic examination, was homogenized for respiration studies in the Warburg apparatus. The results obtained on the changes in enzymatic activity (*22*) will be reported in detail at a later date.

Routes of Administration

A few exploratory experiments with podophyllotoxin and alpha-peltatin were performed to examine the relative effectiveness of various routes of administration in inducing tumor damage. Table 5 presents the data obtained with the intravenous and oral routes. A straight, or slightly curved, blunt 18-gauge needle was inserted in the esophagus for the oral administration; the materials were given in a volume of 0.01 cc. per gram of body weight. In the rare instances in which lacerations of the esophagus were seen at autopsy, the mice were discarded. Intravenous injections were given in the lateral tail vein.

On comparing table 5 with table 3, it is seen that a higher dose was required *via* the oral route than *via* the subcutaneous route for induction

Table 5.—*Effect of other routes of administration on inducing damage in Sarcoma 37 with podophyllotoxin and alpha-peltatin*

Compound	Route	Dose	Mortality[a]	Killed after	Induced tumor damage[b]
		μg./gm.		*Hours*	
Podophyllotoxin	Oral	[c] 100	2/5	8	3/3
	"	[c] 50	5/6	24	1/1
	"	[c] 50	0/8	168	8/8
	"	[c] 30	0/8	24	0/8
Alpha-peltatin	Oral	150	3/8	168	5/6
	"	[c] 100	0/5	8	5/5
	"	100	3/7	168	4/4
	"	75	0/8	24	5/8
	"	50	0/8	24	5/8
	"	25	0/8	24	0/8
	Intravenous	5	0/8	24	8/8
	"	2	0/8	24	8/8

[a] Expressed as: $\frac{\text{Mice dead}}{\text{Mice treated}}$.

[b] Expressed as: $\frac{\text{Number of mice with induced tumor damage}}{\text{Number of mice sacrificed}}$. The tumors in untreated controls showed no induced damage (see p. 1275).

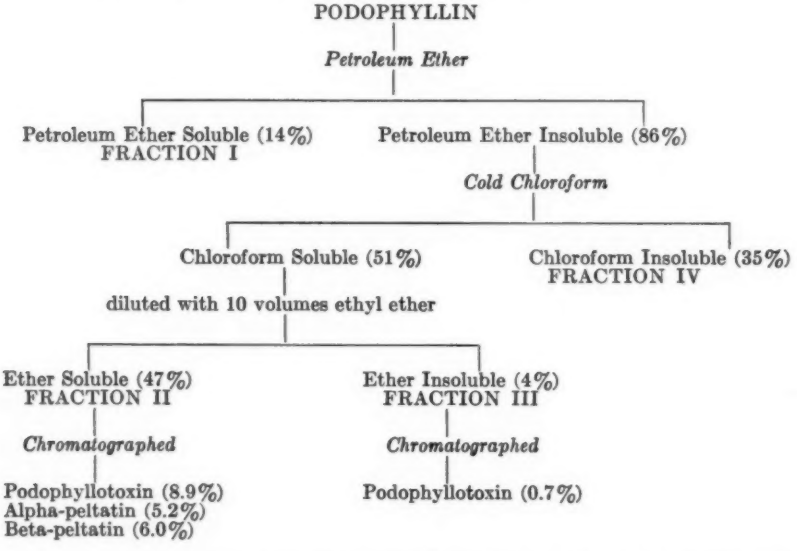
[c] In olive oil; all others were in aqueous solution.

of damage to the tumors. The minimum effective dose orally was about 50 micrograms per gram for both compounds, *i. e.*, more than 10-fold greater than the MED *via* the subcutaneous route. Moreover, the range between MED and MTD *via* the oral route was much smaller than *via* the subcutaneous. The limited data on intravenous administration suggest that this route is at least as effective as the subcutaneous in inducing damage to the tumors with alpha-peltatin.

Search for Additional Active Components

At the outset, damage to Sarcoma 37 was used as an indicator for isolation of those constituents of the crude drug which had potency in damaging tumors. In this way, three highly active compounds were isolated, only one of which was found to be identifiable. The systematic fractionation was continued with solvent partition and chromatography in an attempt to isolate any other highly potent components that might be present. Thus far, no such additional substances have been found. A few of the salient features of that investigation [6] are summarized here.

Crude podophyllin was extracted in the following manner:



A preliminary extraction with petroleum ether yielded a greenish oil (Fraction I) which contained the oil responsible for the odor of podophyllin. The residue from this extraction, upon treatment with cold chloroform, gave a soluble portion and an insoluble one (Fraction IV). The chloroform soluble portion was diluted with about 10 volumes of ethyl ether, whereupon a moderate amount of material precipitated out. The ether soluble material (Fraction II) contained the bulk of the toxin

[6] Unpublished experiments of Detty, Hartwell, and Leiter

and the two peltatins; the ether insoluble portion was designated Fraction III.

The tumor-damaging potency of these fractions was determined by the methods described above. From the relative proportions in the crude drug of the various active fractions, and from the MED of each of them, the summated activity was compared with that of the unfractionated drug. These calculations are given in table 6 which shows that the sum of the activities of the fractions (<105 percent) was the same, within the experimental error, as that of the crude drug. Most of the activity (94 percent) was contributed by Fraction II, the one that contained the bulk of the podophyllotoxin and of the two peltatins. Fraction III, the only other which possessed appreciable activity, was found to contain approximately 20 percent podophyllotoxin; this accounted for about one-third of the activity of this fraction. When this fraction was dissolved in cold alkali, which destroys the potency of podophyllotoxin, the MED was approximately 25 micrograms per gram, indicating the possible presence of alpha-peltatin and beta-peltatin, in an amount of about 10 percent, or of some unknown active compound or compounds.

TABLE 6.—*Tumor-damaging potency of the fractions of podophyllin*

Fraction	Amount Found in Crude Drug	MED	Proportion of Total Activity [1]
Podophyllin (unfractionated)	*Percent* 100	*μg./gm.* 10	*Percent* 100
I. Petroleum ether soluble	14	1,000	<1
II. Chloroform soluble—ether soluble	47	5	94
III. Chloroform soluble—ether insoluble	4	5	8
IV. Chloroform insoluble	35	250	<2
Total	100		<105

[1] Calculated from reciprocal of the MED and the percentage of each fraction found in the crude drug.

These calculations suggest that most of the activity of crude podophyllin may be ascribed to the three highly potent compounds. The quercetin, which has a low order of activity, is insoluble in chloroform and therefore would be contained in Fraction IV, which accounts for less than 2 percent of the potency of the whole drug. Any other active components, if present, would be likely either to have low activity or to be present in low concentration.

Table 7 lists the relative proportion of the activity of the whole drug that was found in each of the three highly active compounds isolated from one batch of material; the figures for the amounts of these compounds are those obtained by Hartwell and Detty (*16*). The actual content of these compounds in the crude drug probably was higher. In any case, these three compounds accounted for the bulk of the tumor-damaging potency of the crude material. Thus the data on the potency and content, both of the fractions (table 6) and of the pure compounds (table 7), showed that most of the activity resided in these three substances. It needs to

be emphasized, however, that these numerical values for biological activity do not have the precision of chemical analysis and that the figures in the last two columns of tables 6 and 7 are only approximations.

TABLE 7.—*Proportion of tumor-damaging activity of podophyllin accounted for by three highly potent constituents*

Compound	Amount isolated from crude drug	MED (approximate)	Proportion of total activity [1] (approximate)
	Percent	*μg./gm.*	*Percent*
Podophyllotoxin	9. 6	2	48
Alpha-peltatin	5. 2	2	26
Beta-peltatin	6. 0	2	30
Total [2]	20. 8	--------	104

[1] Calculated from reciprocal of the MED and the percentage of each compound isolated from the crude drug.
[2] Quercetin is not included here for it contributes less than 2 percent to the activity of the crude drug (see p. 1283).

VEHICLES; SOLUBILITY; EFFECT ON POTENCY AND ON CHEMICAL PROPERTIES

Some data were secured on solubility in various vehicles, and on the effect of aqueous NaOH upon potency and stability. Table 8 gives the solubilities of the three highly active compounds in five solvents. They were most soluble in acetone, propylene glycol, and ethyl alcohol; however, these solvents are not ideal for injection purposes. It was found possible to keep appreciable quantities of these substances in aqueous solution when the propylene glycol, alcohol, or acetone solutions were diluted with as much as four or five volumes of water; these preparations were convenient for injection experiments. Inasmuch as propylene glycol was well tolerated by the mice upon injection of a 50 percent solution in water, relatively concentrated solutions of the three potent compounds were available for injection as solutions in 50 percent aqueous propylene glycol. Both peltatins had only a slight solubility in oil. Nevertheless, suspensions of the peltatins in olive oil exhibited about the same potency in damaging Sarcoma 37 as did their alkaline aqueous solutions.

TABLE 8.—*Approximate solubilities of podophyllotoxin, alpha-peltatin and beta-peltatin in various vehicles*

Vehicle	Podophyllotoxin	Alpha-peltatin	Beta-peltatin
	gm./100 cc.	*gm./100 cc.*	*gm./100 cc.*
Water	0. 01	[a] 2	[a] 2
Ethyl alcohol [b]	[a] 20	2	1. 7
Acetone	>25	10	10
Propylene glycol	5	2. 5	0. 8
Olive oil	0. 5	<0. 1	<0. 1

[a] In 0.1N aqueous NaOH.
[b] Absolute alcohol.
[c] The solubility in 50 percent aqueous alcohol is about 2 percent.

The solubility of the two peltatins in water or in salt solutions was found to be too low for the biological experiments. However, because of their phenolic hydroxyl groups, they could be dissolved in aqueous sodium hydroxide. In the presence of a sufficient excess of alkali, 5 percent solu-

tions of the peltatins were obtained. The excess alkalinity, however, caused sloughing. For concentrations yielding, in convenient volumes, doses of solute not higher than the MTD, less than 0.05 N alkali was necessary for complete solution. Working in dose ranges between the MED and MTD, even less alkali was required, and sloughing was not a prominent feature following a single subcutaneous injection.

The use of alkaline aqueous solutions of the peltatins required some precautions. It was found that alkali converts them to diastereoisomers (*16*) of greatly reduced biological activity (*23*). Table 9 shows the effect upon potency and melting point of allowing a 2 percent solution of alpha-peltatin to remain at room temperature in the presence of 0.1 N sodium hydroxide. In 1 day about four-fifths of the activity was lost; after 1 week about nine-tenths of the activity had disappeared. Heating a fresh solution to 100° C., cooling, and immediately testing for potency, showed that this temperature destroyed practically all the activity in a few minutes. The melting points of the materials recovered by acid precipitation indicated that conversion to the inactive diastereoisomer had occurred. It has been our practice, therefore, to prepare the solutions immediately before injection and to complete the use of the solutions within 10 minutes.

TABLE 9.—*Effect of 0.1 N NaOH on the potency and melting point of alpha-peltatin*

Treatment [1]		MED	MTD	Melting Point [2]
Time of exposure	Temperature			
		μg./gm.	μg./gm.	°C.
10 minutes	Room	2	40	215–225
1 day	Room	10	200	275–276
7 days	Room	20	400	267–274
2 minutes	b. p.	100	1,000	275–276

[1] Of a 2 percent solution of alpha-peltatin.
[2] The initial m. p. of the preparation employed was 215–225° C. This column gives the melting points of the crude precipitates obtained on acidification.

DISCUSSION

The report of Kaplan was soon followed by other investigations of podophyllin for its effects upon benign growths. King and Sullivan demonstrated that it produced changes in the cells of normal skin similar to those produced by colchicine. Thereupon, fractionation of the crude drug was undertaken in this laboratory; two crystalline compounds, which had high potency in causing damage to malignant tumors in mice, were isolated. One of these was identified as podophyllotoxin, while the other was an unknown component at first designated (*1*) as "NCI No. 1074" and later named alpha-peltatin. The following year another new crystalline compound with high tumor-damaging potency was isolated from podophyllin (*11*); this was named beta-peltatin.

Workers at the Sloan-Kettering Institute have reported (*9*, *24*) a selective effect of crude podophyllin upon malignant cells in tissue culture. Their experiments originated from an accidental finding of toxic effects

following the use, in tissue culture experiments, of what was presumed to be normal placental serum, but which was later found to have been procured from a pregnant patient treated with podophyllin for venereal warts. Confirmatory *in vivo* studies were mentioned (*24*) but details were not given.

Two other *in vitro* techniques, using podophyllin, were employed (*25*, *26*) at the Sloan-Kettering Institute on the effect of the crude drug. In one, Sarcoma 180 was grown on the chorio-allantoic membrane of the chick embryo in which the drug was injected; in the other, tumor fragments were immersed in an extract of the drug and then implanted into animals. The former yielded negative results; in the latter, growth of the tumors was inhibited. Only a slight inhibitory effect was noted upon administration of podophyllin to mice bearing spontaneous breast carcinomas.

On the other hand, both in Belkin's (*8*) experiments and in those of this laboratory, striking effects were produced in malignant tumors on treatment of tumor-bearing mice with podophyllin.

Little has been published on the effects of podophyllotoxin upon tumors. Shortly after the report of Hartwell and Shear it was stated by Sullivan and co-workers that podophyllotoxin had an effect on venereal warts similar to that of the crude drug (*27*). More recently they have published some of the experimental details (*28*). Although Karnofsky, in his review (*29*), stated that Belkin had obtained hemorrhage in mouse tumors with podophyllotoxin, this compound was not used by Belkin (*8*, *30*). The previously cited papers from the Sloan-Kettering Institute reported entirely negative, or only slightly positive, findings with podophyllotoxin; these were *in vitro* experiments; *in vivo* results with the toxin were not mentioned.

Despite these essentially negative findings, we have in this laboratory continued to obtain striking damage in tumors following administration of podophyllotoxin to mice. In fact, the toxin was several hundred percent more active than podophyllin in our experiments.

Both alpha-peltatin and beta-peltatin have also proved to be highly potent in producing striking damage in tumors. The peltatins have the advantage of being soluble in alkaline aqueous solution by virtue of the phenolic hydroxyl groups which they contain. The MTD of these compounds in mice appeared to be somewhat higher than that of podophyllotoxin.

As in the case of colchicine, there is little information available as yet on the mode of action of these compounds. One hypothesis proposed by several investigators with respect to colchicine and bacterial polysaccharides postulates (*31*, *32*) that hemorrhage and stasis are produced in the newly formed capillaries of the tumor by these agents, and that necrosis of the tumor tissue is a consequence of interference with the vascular supply. Although our observations that extensive areas of the damaged tumors were hemorrhagic would superficially seem to support such a hypothesis, it does not constitute an adequate explanation

of the action of these drugs. Hemorrhage was conspicuous by its absence in a variety of other tumors, described in the paper following, in which necrosis was induced. Nor, except at lethal doses, was hemorrhage a feature in lymphatic tissues which were also markedly affected by these drugs (*33*).

Investigations in this laboratory have shown (*22*) that rapid and marked reduction of the cytochrome oxidase activity occurs in tumor homogenates from animals treated with the active compounds from podophyllin. Much smaller effects were observed in the normal tissues of the same animals.

In preliminary experiments (*34*) on the fate and distribution of podophyllotoxin in animals, the drug was no longer detectable in the blood or other tissues a few hours after injection.

Tables 6 and 7 indicate that most of the potency of podophyllin may be attributed to the activity of podophyllotoxin, alpha-peltatin, and beta-peltatin; however, fractionation of the crude drug is being continued in the search for still other active compounds which may be present in small amounts. Sullivan recently suggested (*35*) that the effects obtained with aqueous extracts of podophyllin by Rhoads, Sullivan, and Wechsler and by Cornman, on tissue cultures, on onion root tips and on arbacia egg cleavage, may indicate the presence of water-soluble substances other than podophyllotoxin with biologic activity of a similar type. The peltatins have extremely low solubilities in distilled water ($<$0.001 percent); we have not been able to induce damage in S–37 with injections of as much as 1.0 cc. of their saturated aqueous solutions. Despite the low solubility of podophyllotoxin in water (table 8), we have been able to induce damage in S–37 by subcutaneous injection of 0.2 to 0.5 cc. of a saturated aqueous solution (table 2). Since the water solubility of the toxin is high enough to account for the activity of aqueous extracts of podophyllin, further work is required before it can be concluded that additional water-soluble, potent substances other than the toxin are present. Quercetin, the fourth substance known to be present in podophyllin, was active only at doses about 100 times that of the other three known components (table 2), and only when dissolved in aqueous NaOH.

In some recent papers (*27*, *28*), as well as in the older literature (*13*), picropodophyllin and podophyllic acid have been listed, erroneously, as components of podophyllin. Both of these compounds are alkali conversion products of podophyllotoxin. In this laboratory these two substances have induced mitotic arrest at high dose levels; however, no tumor damage was seen grossly (*4*).

Since podophyllotoxin, alpha-peltatin, and beta-peltatin have all demonstrated high potency in damaging Sarcoma 37 at a dose well below the maximum tolerated, more extensive investigations were carried out with these compounds. The response of different types of transplanted tumors is presented in the following paper (*36*). The effect of their derivatives upon tumors is also under investigation (*23*). King (*37–39*) has recently described the pathologic changes in the skin of the mouse

following local application of podophyllin. Experiments on the acute and chronic toxicity (*40*) and the histopathologic effects (*41*) produced by parenteral administration of the highly potent components are in progress at this Institute.

SUMMARY

In an investigation in which some 3,000 mice bearing Sarcoma 37 were employed, it was found that:

(1) Four compounds present in podophyllin accounted for most of the tumor-damaging activity of the crude drug.

(2) Podophyllotoxin, alpha-peltatin, and beta-peltatin were highly potent; quercetin was also active, but was required in a much larger dose.

(3) The minimum effective dose, for a single subcutaneous injection, was about 2 micrograms per gram of body weight for the three highly potent compounds.

(4) The maximum tolerated dose, in a single injection, was in the range of 30 to 40 micrograms per gram for these three compounds.

(5) Tumor damage was observed grossly as early as 6 hours after injection.

(6) Intravenous and oral administration produced similar damage to Sarcoma 37, but much higher doses were required *via* the oral route.

ADDENDUM

Since this paper was received for publication, a revised formula for podophyllotoxin has appeared. [See Hartwell, J. L., and Schrecker, A. W.: Am. Chem. Soc. 72:3320–3321 (1950).]

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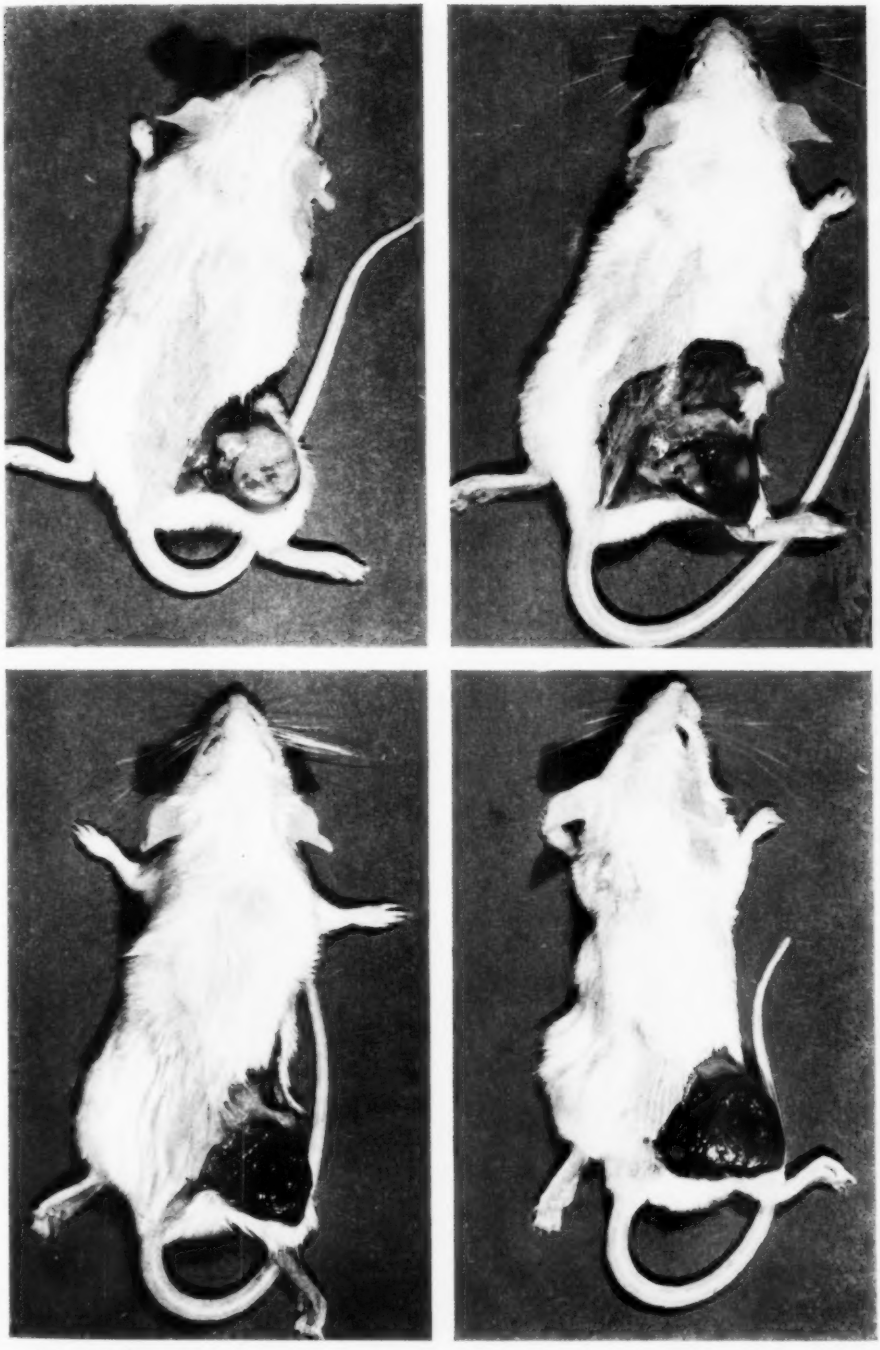
PLATE 187

FIGURE 1.—Gross damage induced in Sarcoma 37 following a single subcutaneous injection of podophyllotoxin (20 μg. per gm. of body weight) in mice bearing 6-day-old tumor implants.

Upper left, untreated control.
Upper right, 6 hours after injection.
Lower left, 24 hours after injection.
Lower right, 48 hours after injection.



Leiter *et al.*

PLATE 188

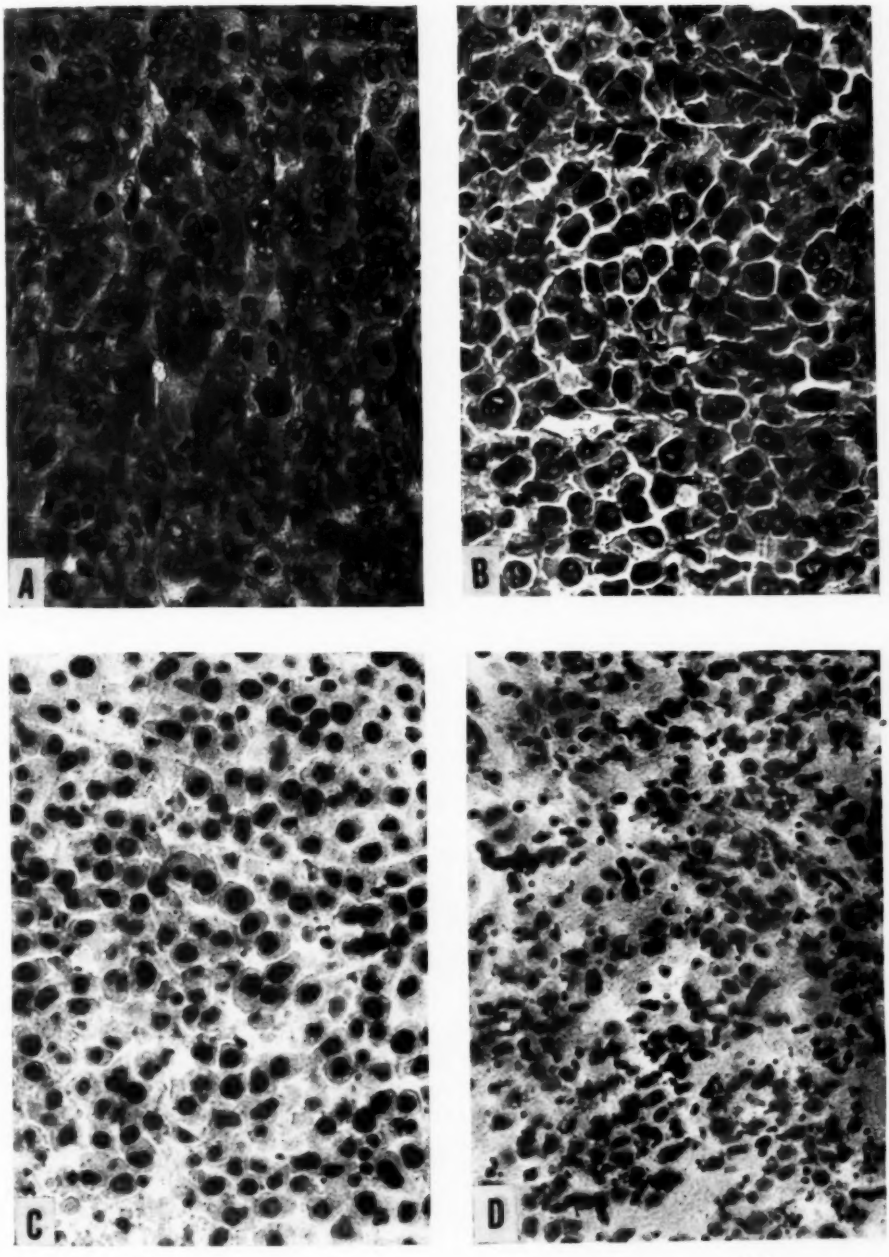
FIGURE 2.—Microscopic damage induced in Sarcoma 37 following a single subcutaneous injection of alpha-peltatin (20μg. per gm. of body weight) in mice bearing 6-day-old tumor implants. Hematoxylin and eosin stain. ×500.

A, untreated control.

B, 4 hours after injection.

C, 24 hours after injection.

D 72 hours after injection.


A
B
C
D

EFFECT OF ALPHA-PELTATIN, BETA-PELTATIN, AND PODOPHYLLOTOXIN ON LYMPHOMAS AND OTHER TRANSPLANTED TUMORS [1] [2]

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The various findings with podophyllin stimulated the investigation of the chemical and biological properties of the crude drug described in the preceding paper (*2*). The recent interest in podophyllin arose out of clinical observations; in 1942, Kaplan (*3*) described the remarkable efficacy of podophyllin when applied locally to human condylomata; this therapeutic practice was said (*4*) to have been generally familiar for some time to urologists in New Orleans. Kaplan's observations were soon confirmed, and extended, in other investigations (*5–10*); a favorable response to topical podophyllin therapy has also been reported for plantar warts (*11*), soft papillomas of the female urethra (*12*) and bladder papillomas (*13*).

More than 75 years ago, severe ulceration of the cornea (*14*) and dermatitis (*15*) were observed in workers engaged in grinding podophyllin root and in preparing its resinous extracts. For many years podophyllin has been employed as a constituent of proprietary remedies for its cathartic and alleged cholagogic action, but claims that it has significant activity as a cholagogue have not been substantiated (*16*).

In this laboratory it had been found (*17*) that podophyllotoxin manifested high potency in producing damage in mouse tumors; moreover, two additional compounds of high potency were also isolated (*18–20*), and some potency was found (*2*) to be possessed by quercetin as well. Sullivan and coworkers (*9, 21, 22*) have stated that podophyllotoxin was the active substance in the crude drug responsible for its curative effect on human condylomata, and that quercetin was inactive.

In the preceding studies in this laboratory, the tumor employed was mouse Sarcoma 37. This communication describes the effects of three potent constituents of podophyllin upon several transplanted tumors of rodents including three different lymphoid tumors.

MATERIALS AND METHODS

The alpha-peltatin, beta-peltatin, and podophyllotoxin employed were specimens isolated by Hartwell and Detty (*18–20*). The podophyllotoxin was administered in olive oil solution. The peltatins were dissolved in

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[2] Presented, in part, at the Detroit meeting (*1*) of the American Association for Cancer Research, April 16, 1949.
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approximately 1 N NaOH (0.1 cc. per 10 mg. of compound), and diluted with water; the volume was adjusted so that 0.01 cc. usually contained the dose desired for each gram of body weight. In this way the total volume injected was readily calculated for each mouse from its body weight, and was usually limited to 0.2–0.3 cc. for mice weighing between 20 and 30 grams.

The following mice were used: strains dba (subline 212), A, and C3H (Heston subline); CAF_1 hybrids (♀ strain C × ♂ strain A); ABC backcrosses [strain A × F_1 (strain A × strain C3H)]. The mice at the beginning of the experiments were 2 to 4 months of age, weighed 18 to 25 grams, and were of both sexes. The rats were of the Buffalo strain. Purina chow and fresh vegetables constituted the diet.

In transplanting (*2*) the tumors, about 0.05 cc. of a tumor mash was injected intramuscularly in the right thigh. The tumors used were:

A) *Leukemia 1210*, an acute stem-cell leukemia induced in dba mice with methylcholanthrene by Law (*23*). Local tumors larger than 2 cc. in volume developed in 1 week and continued to grow rapidly; this was accompanied by regional adenopathy, blood stream invasion, and visceral infiltration. In untreated mice the terminal blood picture was characterized by marked leukocytosis (40,000–200,000 per cubic millimeter), large numbers of stem cells (5 to 40 percent) in the peripheral blood, moderate polychromatophilia, and moderate anemia. Hemorrhage was not usually observed in the tumors or in the normal tissues. In 172 control animals of 17 tumor transplant generations, survival after implantation varied between 10 and 17 days, with an average of 12 ± 1 days. The implants grew in almost all host mice of line 212 of the dba strain. No spontaneous regressions were observed in some 800 mice bearing this tumor.

B) *Lymphosarcoma 1*, a localized tumor originally induced by Lorenz (*24*) in a strain A mouse following exposure to x-rays. Palpable tumors developed from implants in almost all mice of the A, CAF_1, and ABC stocks in 12 to 14 days. The mass at the inoculation site grew to a volume of about 20 cc. by the time the animals died; no metastases were detected grossly. Survival time after tumor implantation in 76 untreated animals of 8 tumor generations varied from 26 to 34 days with an average of 30 ± 3 days; none of these tumors regressed. In untreated mice the tumors exhibited a negligible amount of cystic degeneration and necrosis during the first 3 weeks after implantation, in spite of their enormous size.

C) *Lymphosarcoma 2*, a metastasizing tumor, also induced with x-rays in a strain A mouse by Lorenz (*24*). Palpable growths appeared 10 to 12 days after inoculation, and grew progressively, in almost all A, ABC, and CAF_1 mice. A large solitary, regional (periaortic) lymph node usually developed. In the later stages of tumor growth, massive infiltration of the spleen was seen grossly; microscopically, infiltration of the liver was also observed. In 49 untreated mice of 5 tumor generations, survival varied from 14 to 24 days with an average of 17 ± 1 days. Spontaneous regression was not seen in some 200 untreated mice bearing this tumor.

D) *Mammary adenocarcinoma C3HBA*, a tumor that had developed

spontaneously in a C3H mouse of the Andervont subline, and which had been transplanted (*25*) for about 50 generations. Tumors developed in almost all C3H mice of the Heston subline, and in about half of the Bar Harbor subline; in 10 to 12 days after inoculation the tumors were large enough to be readily evident. Only a small amount of necrosis and cystic degeneration was observed during the first 3 weeks of tumor growth. Even when the tumors were large, the acinar structure was well preserved. Spontaneous regressions were not observed in some 100 untreated mice bearing this tumor.

E) *Melanoma S-91*, a tumor which originated in dba mice in experiments of Cloudman (*26*). The tumors were evident 12 to 15 days after implantation, and growth was satisfactory in all of the dba mice (more than 200). About 60 untreated mice with this tumor survived 30 to 50 days; spontaneous regressions were not observed.

F) *Rat Carcinoma 1643*, a tumor originally induced in a Buffalo rat with N-acetyl-2-aminofluorene by Morris and coworkers; upon transplantation it gave rise (*27*) to 2 histologic patterns, *viz.*, a polymorphic carcinosarcoma composed of spindle-shaped cells and large irregular polygonal cells in an abundant loose intercellular stroma. Growths developed 10 to 12 days after inoculation of tumor fragments, and reached 50 to 100 cc. in size. Spontaneous regressions were not observed in some 30 untreated animals with this tumor; they succumbed in 45 to 60 days, without evidence of metastasis.

The agents were administered subcutaneously, in the axillary space contralateral to the tumor. The injections were usually begun when the intramuscular tumors became grossly detectable. However, some of the survival experiments with Leukemia 1210 were initiated as early as the third or fourth day after implantation, prior to the appearance of the local tumor mass; even in this instance only about 1 week was available for treatment inasmuch as untreated mice bearing this tumor succumbed in about 12 days.

For each experimental group there was a comparable group of untreated control animals bearing implants of the same tumor generation. Data were recorded on body weight, approximate tumor size, and survival. Maximum tolerated dose (MTD) and minimum effective dose (MED) are terms employed in the same sense as in the preceding paper (*2*). Standard hematological methods were used in the examination of peripheral blood and bone marrow. Damage induced in the tumors was evaluated from their gross and microscopic appearance on comparison with tumors of untreated control mice, of the same experiment, sacrificed at the same time. In addition, neoplastic and normal tissues were examined at various intervals after single and repeated administration of the drugs; the tissues were fixed in Zenker-formol solution, and the sections stained with hematoxylin and eosin. The pathological changes produced with these agents in normal tissues of laboratory animals are being studied (*28*). Toxicological and pharmacological observations (*29*) will be reported in detail in a later communication (*30*).

RESULTS

Gross Changes in Tumors

Acute damage was induced in all types of tumors studied within 24 hours after a single subcutaneous injection of any of the highly active components of podophyllin. In general, similar experiments were performed, and analogous results were obtained, with alpha-peltatin, beta-peltatin, and podophyllotoxin. For brevity, however, only illustrative data are given, taken chiefly from the experiments with alpha-peltatin, the compound most extensively used.

On gross inspection of transverse sections of the tumors, the acute damage was evident from the severe caseous necrosis and partial liquefaction. In some types of tumors extensive hemorrhage was also induced. The damage could be seen grossly as early as 6 or 8 hours in most instances, as had previously been observed in Sarcoma 37. There was variation in the response of different types of tumors to the same dose of a given compound. With the same type of tumor, the response varied with the dose of the agent and with the size and age of the tumor. With multiple injections, these same factors were important; in addition, previous treatment altered the response as described below. Microscopically, the findings paralleled the gross observations.

Intraperitoneal or intravenous injection produced changes similar to those obtained with the subcutaneous route. Complete regression was not observed in any of the experiments described in this paper; all animals died with evidence of tumor.

Under specified conditions, the tumors in treated mice were appreciably smaller than in untreated controls; the phrase "retardation of tumor growth" connotes this type of observation. The findings observed with the various kinds of transplanted tumors were as follows:

Lymphomas: Within 24 hours after injection these tumors acquired a yellowish-gray, caseous necrotic appearance. During the next 24 hours, some liquefaction and slight shrinkage of the tumors were observed (figs. 1 and 2). On the 2d post-injection day, the external portions of these tumors frequently showed a diffuse, reddish-black discoloration. (This is not to be confused with the intense hemorrhage and necrotic reaction in Sarcoma 37, and in some other tumors, which develop in about 6 hours following the injection of some chemical agents; in such cases the hemorrhage occurs within the tumor mass.) After 3 or 4 days, growth of the tumors was resumed, apparently from the margins of the dried, blackened, shrunken tumors. In the case of Lymphosarcoma 2, the metastases responded as did the primary tumors.

After a single dose, tumor growth was retarded for 24 to 72 hours; illustrative data for the three lymphomas are given in text-figures 1 to 4. Text-figure 1 shows the effect of alpha-peltatin on the size of Leukemia 1210 and Lymphosarcoma 1. Initially, the tumors in the test groups were approximately the same size as those in the controls. Column 3 in this chart gives the day of tumor growth when each injection was

given; the size in the last column represents the average for each group of the approximate tumor volume measured on the day stated in column 4. At a minimum effective dose (2.5 μg. per gm.) no difference in tumor size resulted; with larger doses, appreciable differences were obvious. In general, a greater difference in size between the test and control tumors was obtained with multiple injections (text-figs. 1, 3, 4; and fig. 3).

EFFECT OF ALPHA-PELTATIN ON VOLUME OF LYMPHOID TUMORS

Tumor	Dose	Time after implanting		Number of Mice	Tumor volume cc. (Average) 2.5 5. 7.5 10.0
		Treated	Measured		
	µg/gm	Days	Days		
Leukemia #1210	2.5	7	11	6	
"	—	Control	11	6	
"	20	5	8	8	
"	—	Control	8	8	
"	40	4	8	6	
"	—	Control	8	6	
"	10	4, 6, 8	10	13	
"	20	4, 7,	10	12	
"	—	Control	10	13	
Lymphoma #1	20	14	17	8	
"	—	Control	17	7	
"					
"	20	14	21	10	
"	20	14, 18	21	10	
"	10	14, 17	21	10	
"	10	14, 16, 18, 20	21	12	
"	—	Control	21	14	

TEXT-FIGURE 1.—Effect of alpha-peltatin on tumor size.

RESULTS

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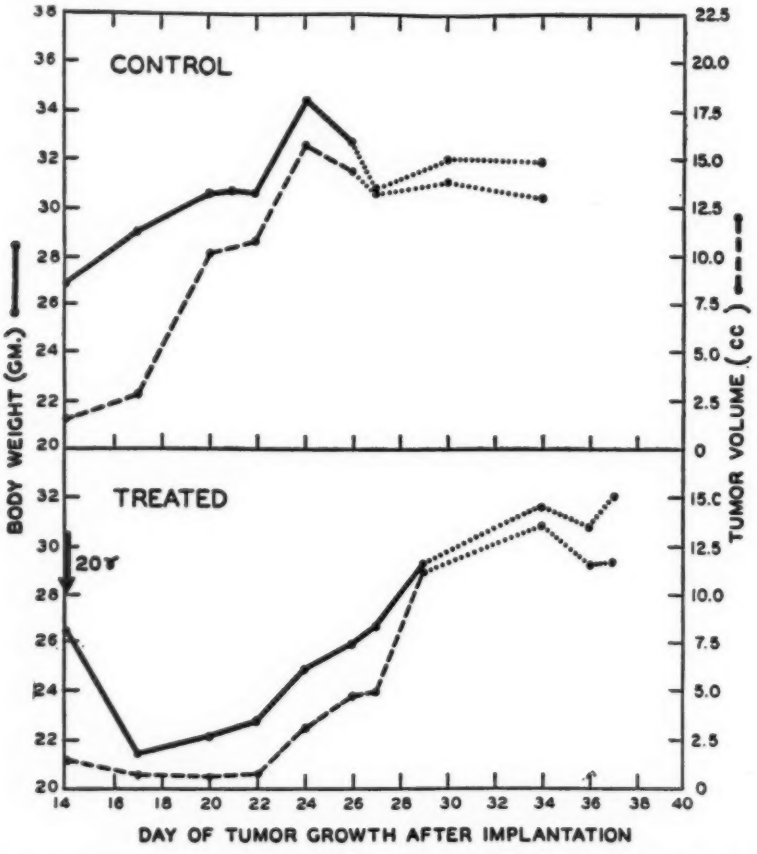
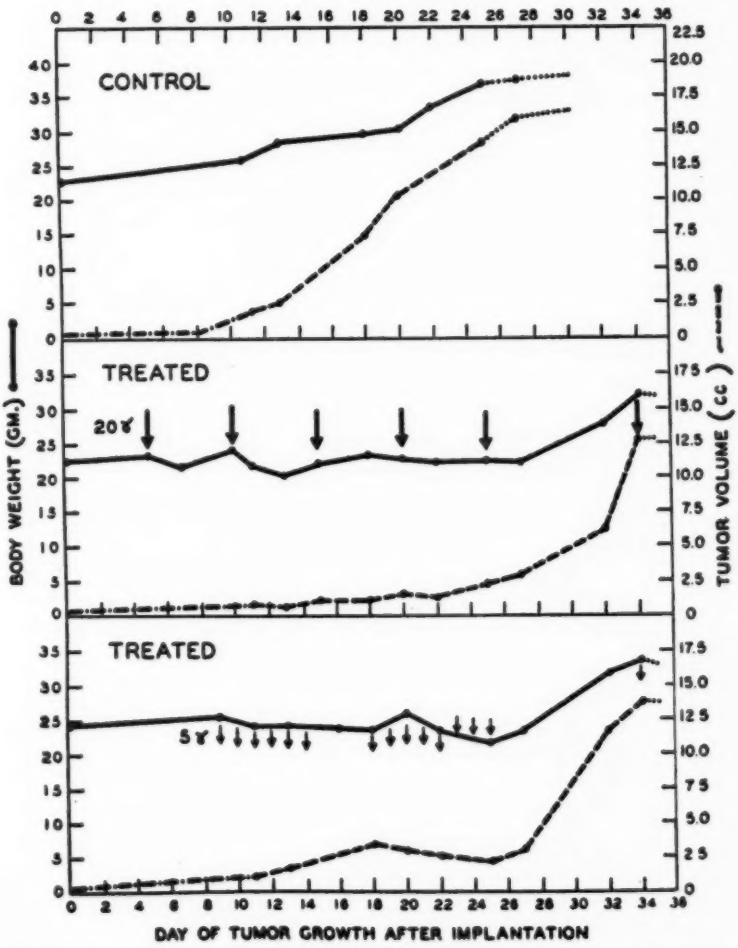
		Treated	Measured		2.5 5. 7.5 10.0
	μg/gm	Days	Days		
Leukemia #1210	2.5	7	11	6	
"	—	Control	11	6	
"	20	5	8	8	
"	—	Control	8	8	
"	40	4	8	6	
"	—	Control	8	6	
"	10	4, 6, 8	10	13	
"	20	4,7,	10	12	
"	—	Control	10	13	
Lymphoma #1	20	14	17	8	
"	—	Control	17	7	
"					
"	20	14	21	10	
"	20	14,18	21	10	
"	10	14,17	21	10	
"	10	14,16,18,20	21	12	
"	—	Control	21	14	

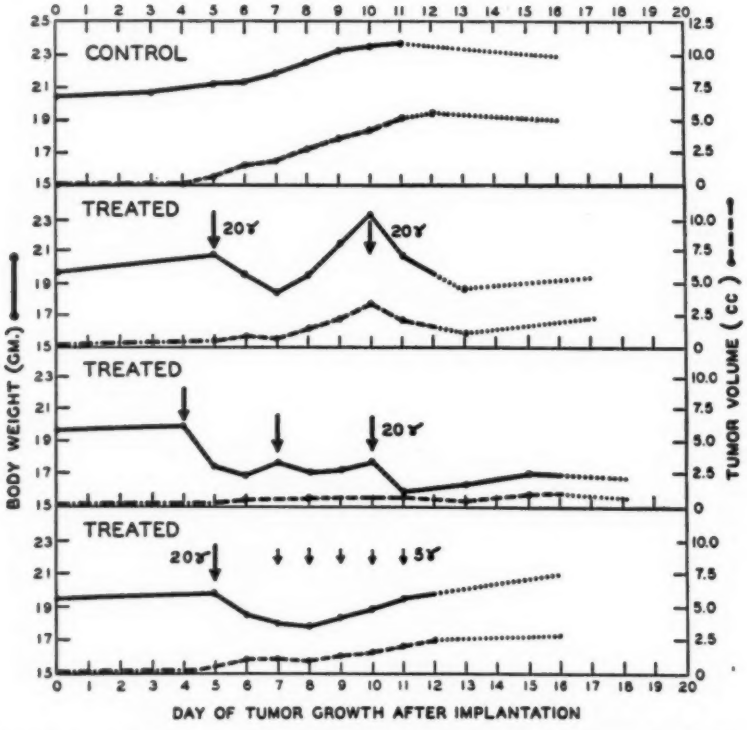
TEXT-FIGURE 1.—Effect of alpha-peltatin on tumor size.



TEXT-FIGURE 2.—Effect of a single subcutaneous injection of alpha-peltatin (20 μg. per gm.) upon body weight and tumor volume; strain A mice bearing Lymphosarcoma 1. Data are the averages for six control and six treated mice. Solid lines represent body weight; dash lines, approximate tumor size. After one or more deaths, the data are shown by dotted lines. Injection given 14 days after implantation of tumor.

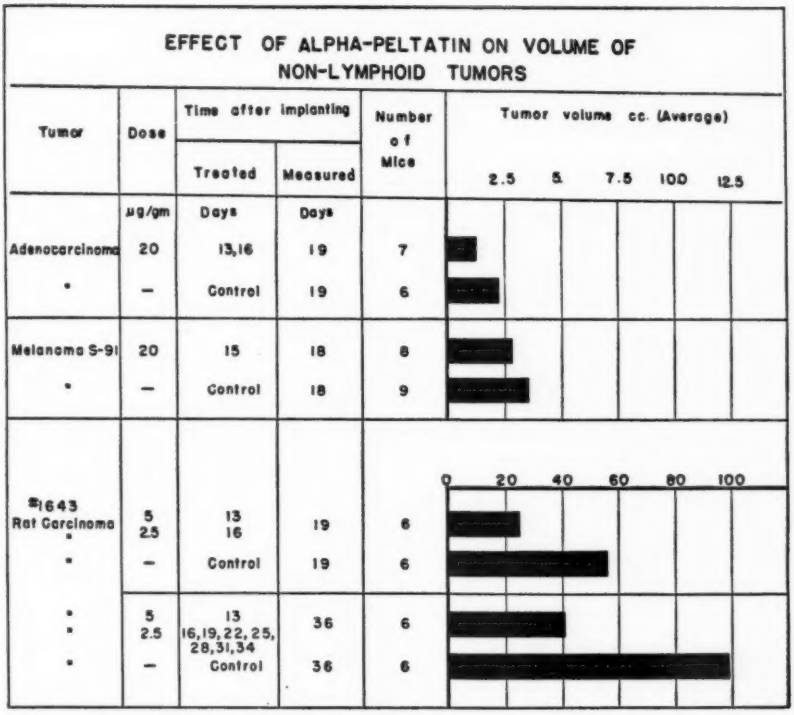


TEXT-FIGURE 3.—Effect of multiple injections of alpha-peltatin upon body weight and tumor volume; strain A mice bearing Lymphosarcoma 1. Each group consisted of eight mice. Lines represent data as in text-figure 2. Treatment during first 26 days resulted in smaller tumors not accompanied by deaths or great loss in body weight. When treatment was discontinued, the tumors grew rapidly. After an 8-day rest, a single injection of the same amount killed all the animals at both dose levels.



TEXT-FIGURE 4.—Effect of alpha-peltatin upon body weight and tumor size; Leukemia 1210 in dba mice. Eight mice per group. Lines represent data as in text-figure 2. Note influence of dose level and of number of injections upon body weight and tumor size.

Other Tumor Types: After a single MTD of alpha-peltatin, hemorrhagic discoloration and some liquefaction were noted in the mouse adenocarcinoma, and in the rat polymorphic carcinoma (fig. 4), during the first day; this was in contrast to the lymphomas which exhibited no induced hemorrhage during the first 24 hours following injection. The melanoma changed after treatment from a firm, dark gray tumor to a partially liquefied, purplish-gray mass. Text-figure 5 shows the effect on tumor size. The retardation in size of the mouse carcinoma and of the melanoma was less than in the case of the other tumors. Even this slight change in the melanoma was brought about only with a dose high enough to result in death of some of the mice.



TEXT-FIGURE 5.—Effect of alpha-peltatin on tumor size.

MICROSCOPIC OBSERVATIONS

As seen microscopically the damage induced in the three different lymphoid tumors with a single injection of alpha-peltatin occurred at dose levels (table 1) similar to those found (*2*) to affect Sarcoma 37. For both alpha- and beta-peltatin, the minimum dose effective in damaging the tumors (MED) was about the same in the case of the leukemia and of the lymphosarcomas. The affected tumor tissue showed changes (figs. 5, 6, 7, 8) similar to those induced with podophyllotoxin and with crude podophyllin (*31*). Within 6 hours after administration of an effective dose (5 to 20 µg. per gm.) of alpha- or beta-peltatin, generalized pyknosis of cells was observed throughout most of the tumor. The next day, induced necrosis was regularly seen; it was manifest from generalized

pyknosis, karyorrhexis, multinucleation, chromatin dust, cytoplasmic vacuolization, eosinophilia of cytoplasm, and widespread loss of cell outline. However, even after high doses a few apparently unaffected cells were usually seen in the peripheral areas of the tumors, adjacent to muscle bundles or blood vessels such as those seen in fig. 6*A*. At the minimum effective dose, the pyknotic and necrotic changes were observed in cells scattered irregularly throughout the tumor (fig. 6*B*); in addition, many of the cells showed distorted mitotic figures, clearer delineation of nuclear outlines, and shrinkage of cytoplasmic margins. Giant cells, presumably malignant, were observed at the periphery during the recovery phase (48 to 96 hours) after a single injection. Large, debris-laden macrophages were often seen in blood vessels traversing affected areas of treated tumors. In contrast to Sarcoma 37, the lymphoid tumors in the untreated control mice showed little central necrosis.

TABLE 1.—*MED and MTD for a single subcutaneous injection of alpha-peltatin in mice with transplanted tumors*

Tumor	Mouse	MED (approximate)	MTD (approximate)
		μg./gm.	*μg./gm.*
Sarcoma 37 *	CAF_1	2	40
Leukemia 1210	dba	2	40
Lymphosarcoma 1	ABC	2	40
Lymphosarcoma 2	ABC	2	40
Adenocarcinoma C3HBA	C3H	15	40
Melanoma S–91	dba	20	20

MED—Minimum effective dose.
MTD—Maximum tolerated dose.

* Data from preceding paper (*8*).

In the adenocarcinoma and in the melanoma, a single dose of alpha-peltatin produced a lesser degree of damage; for a minimal effect in these tumors, doses were required that were up to ten times as large (15 to 20 μg. per gm.) as the minimum effective dose in the lymphoid tumors (2 μg. per gm.). In the melanoma, a maximum tolerated dose produced pyknotic and necrotic changes (fig. 9) but only in infarct-like, patchy areas of the tumors. In the mouse adenocarcinoma, a large dose produced generalized pyknosis in most of the acini (fig. 10), although cellular disintegration and nuclear dust were not so striking as in the treated lymphoid tumors. The rat carcinoma also showed generalized pyknosis, but less fragmentation of chromatin than did the lymphomas; the effects obtained in the rat carcinoma with the MTD are illustrated in figure 11.

After tolerated doses hemorrhage was not conspicuous, either grossly or microscopically, in the lymphomas. Blood vessels frequently appeared unaltered within areas otherwise showing frank necrosis. Tumors in mice treated with a minimum effective dose frequently contained blood vessels which were surrounded by cuffs of apparently undamaged tumor cells (fig. 6*A*).

Systemic Toxicity

Animals bearing well-developed lymphomas lost weight following a single injection of either of the peltatins in a dose above the minimum effective one. A single injection of an effective dose of alpha- or beta-peltatin resulted in weight losses (table 2) of about 15 percent during the first 48 hours following treatment. However, such doses produced little or no weight loss in animals without tumors. The weight loss in dba mice with the leukemia was greater than in ABC mice bearing the lymphosarcomas.

Table 2.—*Loss in body weight following a single dose of alpha- or beta-peltatin in dba mice bearing Leukemia 1210*

Mice [a]	Treatment	Number of mice	Dose	Average loss in weight [b]
			μg./gm.	*Percent*
With tumor [c]	Alpha-peltatin	6	2.5	0
" "	" "	8	5	15
" "	" "	12	5 x 4[d]	14
" "	" "	8	10	15
" "	" "	8	20	16
No tumor	" "	10	20	1
" "	" "	8	40	7
With tumor	Beta-peltatin	6	2.5	0
" "	" "	8	10	13
" "	" "	8	20	16
" "	" "	10	40	13
No tumor	" "	10	5	0
" "	" "	8	40	3
With tumor	Starvation [e]	8		17
No "	"	20		28

[a] dba subline 212.
[b] 48 hours after a single injection.
[c] 7 day old intramuscular implants.
[d] 4 daily injections.
[e] No food for 48 hours; no water for 24 hours; no drug given; no deaths occurred.

In the leukemia-bearing mice, weight loss in the 48-hour period following a single effective dose amounted to about 15 percent. An equivalent or greater weight loss was produced both in normal mice and in those bearing the leukemia by deprivation of food for 48 hours and of water for 24 hours. Thus, loss of appetite for food and water may possibly account for most of the weight loss in the treated animals with large tumors. It is obvious, however, that the weight loss following use of these drugs was not responsible for the gross changes in tumor size or for the effect on survival (see below) inasmuch as an equivalent weight loss produced by deprivation of food and water did not give these effects in the tumors. Three or four days after a single injection of these substances, the body weight of the treated tumor-bearing animals began to rise steadily toward that of the untreated controls (text-fig. 2).

Although diarrhea was noted at near-lethal dose levels, it was seen only occasionally at lower doses. A separate communication (*30*) will deal further with the pharmacological properties of these agents.

In the later stages of tumor growth the tolerance of the mice for the peltatins was less. This was evident when the tumors exceeded the following ages: Leukemia 1210, 8 days; Lymphosarcoma 1, and the adenocarcinoma, 16 days; Lymphosarcoma 2, 14 days; and Melanoma S91, 20 days. Thus, the toxicity was dependent not only on the total amount of drug administered, but also upon the age and type of the tumor at the time of injection. Mice in which tumor size had been retarded by previous drug treatment, tolerated subsequent treatment better than animals bearing untreated tumors of comparable age. For example, a dose of 20 μg. per gm., on the 10th day after implantation of the leukemia, was tolerated better by mice which had already received two or more injections of this dose than by animals previously untreated (compare Experiments 8 and 18 in table 3). Thus, within the effective dose range, the repeated administration of a given dose of alpha-peltatin, in mice with small young tumors, was less toxic than a single dose of the same size when given later in the course of tumor development. Repetition of doses within the tumor-damaging range was best tolerated when the injections were spaced at 48- to 72-hour intervals (text-fig. 4 and table 3).

TABLE 3.—*Survival of dba mice bearing Leukemia 1210 after various dose schedules of alpha-peltatin* [a]

Experiment No.	Dose	Day of treatment	Number of mice	Survival	Alteration in survival [b]
	μg./gm.			*Days*	*Percent*
1	2.5	3, 4, 5, 6, 7, 8, 9	10	13±1	+8
2	5	3, 4, 5, 6, 7, 8, 9	10	14±1	+17
3	10	3, 5, 7, 9, 11	15	16±2	+33
4	20	3, 6, 9	14	16±2	+33
5	10	4, 7, 10	25	14±1	+17
6	10	4, 6, 8, 10, 12	38	16±2	+33
7	10	4, 7, 9, 11, 13, 15	8	16±0	+33
8	20	4, 7, 10	51	17±2	+42
9	20	4, 7, 10, 13	8	13±2	+8
10	20	4, 7, 9, 11, 13	10	13±1	+8
11	10	5, 8, 11	24	12±2	+0
12	5	5, 6, 7, 8, 9, 10	16	12±2	+0
13	20	5, 8, 11	19	13±1	+8
14	20	5, 10	8	15±2	+25
15	20 5	5 7, 8, 9, 10, 11	8	15±2	+25
16	20	7	7	16±2	+33
17	10	10	11	12±4	0
18	20	10	10	11±1	−8
8a	(c)	4, 7, 10	9	12±2	0
16a	(c)	7	7	13±1	+8

[a] These experiments are listed in order of increasing age of implants at time injections were started.
[b] Survival of 157 untreated control mice in 10 separate experiments (see text-fig. 6) was 12±1 days.
[c] Deprivation of food and water (see text); no compound injected.

In these experiments the tolerance to single or multiple doses of the peltatins varied with the histologic type, age and size of the tumor, and with the previous treatment. The data on MTD and MED given in table 1 are for a single dose in animals bearing palpable young tumors; ratios of MTD to MED ("therapeutic index") calculated from these data are to be regarded merely as crude guides for further experimental work.

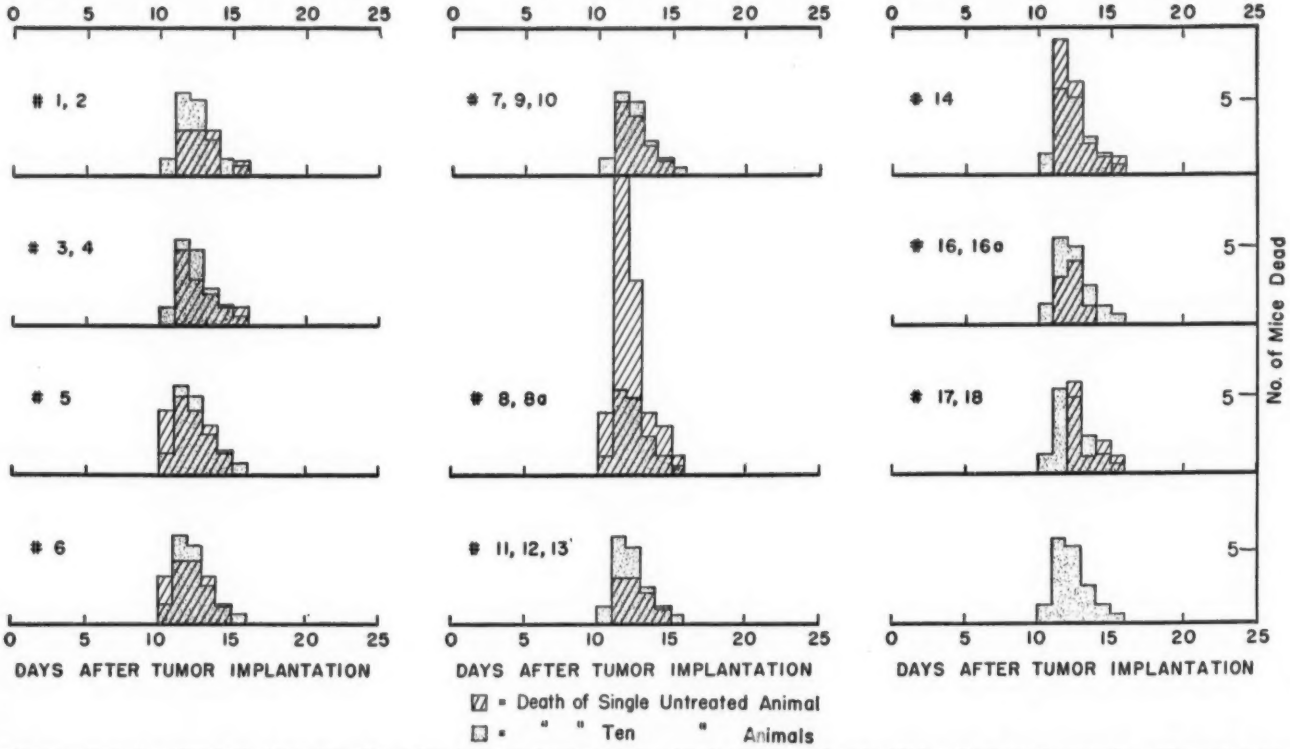
Survival

A slight increase in survival time of animals bearing lymphoid tumors was apparent in experiments with single and multiple doses of the peltatins administered early in the course of tumor growth. Animals with the leukemia showed some prolongation in survival after a single dose of 20μg. per gm. of alpha- or beta-peltatin on the 7th day. After treatment with a maximum tolerated, or a minimum effective dose (40 μg. and 2.5 μg. per gm., respectively) no increase in survival time was observed.

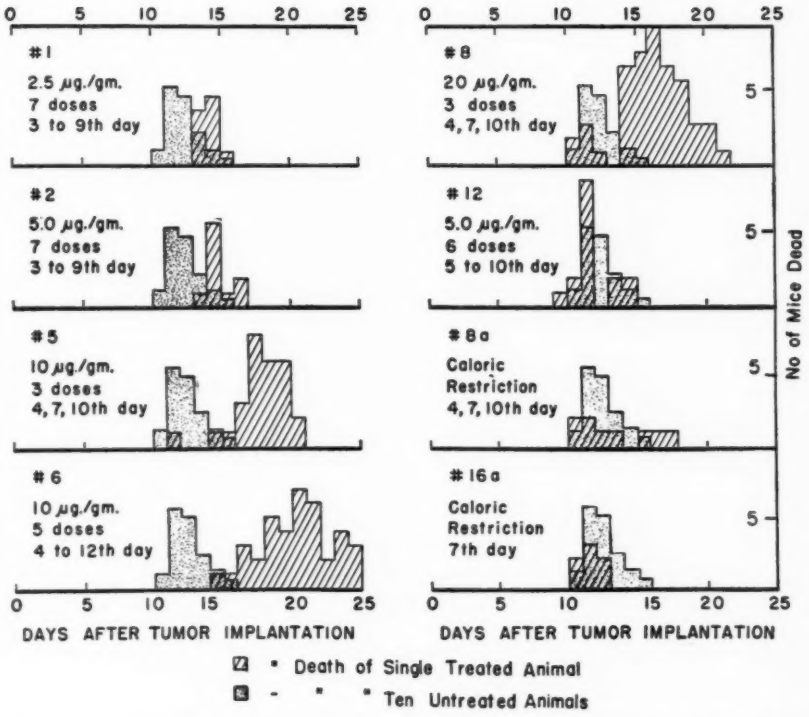
The remarkably high reproducibility in the survival data of dba mice bearing Leukemia 1210 observed by Law and coworkers (*23*) was also obtained in our experiments. Text-figure 6 shows the data in each of 10 control experiments, conducted over a period of several months, in comparison with the composite graph for all these untreated mice combined; the great consistency in the results of the different control experiments is obvious. As a base of comparison with the survival data for the treated groups, the composite control graph was employed.

Several multiple dose schedules of alpha-peltatin were tried in mice with the leukemia. An increase in survival of about 35 percent was obtained with 20 μg. doses given on the 4th, 7th, and 10th days after inoculation, and with 10 μg. doses given every other day during the same period (Experiments 8 and 6 in table 3 and text-fig. 7). Single or repeated injection with the extremes of the dosage range (40 μg. or 2.5 μg.) was either too toxic or ineffectual (text-fig. 8). An increase in survival was also obtained with 10 μg. and 20 μg. doses of alpha- and beta-peltatin in mice bearing either the metastasizing or the nonmetastasizing lymphosarcoma (text-fig. 9).

Treatment with either of the peltatins regularly reduced the survival of mice with the melanoma as compared with the untreated animals. Inasmuch as the peltatins produced an increase in survival in mice of the same strain (dba) bearing the leukemia, the early deaths in the melanoma-bearing mice are not ascribable to any special sensitivity of the dba strain to these compounds. The presence of the melanoma was apparently responsible for the poor survival of the mice after administration of these drugs.

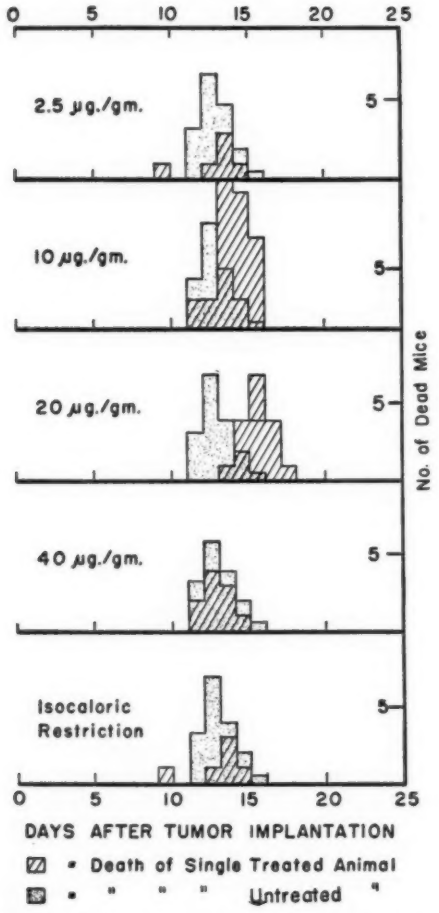


TEXT-FIGURE 6.—Survival data for dba mice bearing intramuscular implants of Leukemia 1210. Untreated controls. The ordinates show the number of mice which died, in each experiment, on the days stated in the abscissae. The lined areas represent data for a single experiment. The combined survival data for all the control experiments are given in the stippled bar graph. Each individual experiment is compared with the summarized data by superposition of the composite graph.

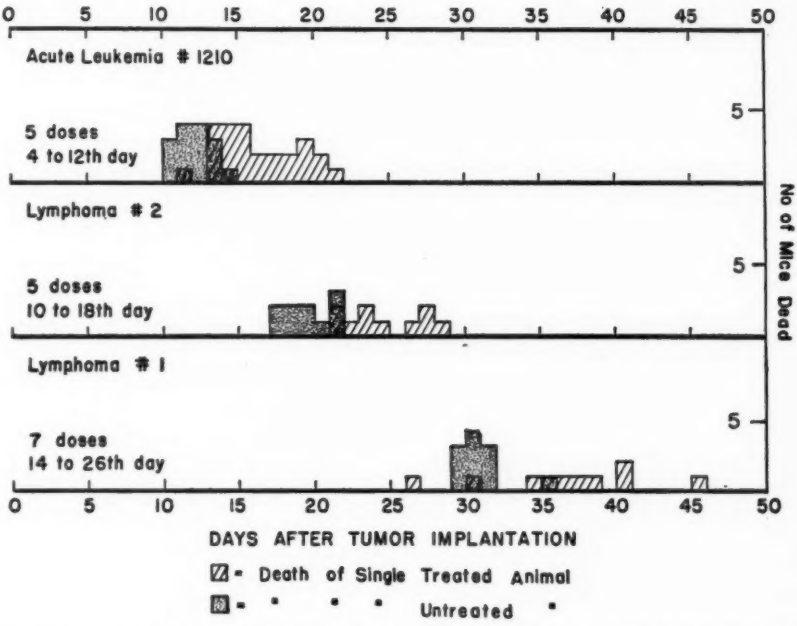


TEXT-FIGURE 7.—Survival data for mice with Leukemia 1210 treated with alpha-peltatin. The results in each experiment are contrasted with the composite data for the untreated controls, shown by the stippled graph.

experiment is compared with the summarized data by superposition of the composite graph.



TEXT-FIGURE 8.—Survival data of dba mice bearing Leukemia 1210 following treatment with beta-peltatin.


0 5 10 15 20 25 30 35 40 45 50
Acute Leukemia # 1210
5 doses
4 to 12th day
5
Lymphoma # 2
5 doses
10 to 18th day
5
Lymphoma # 1
7 doses
14 to 26th day
5
No of Mice Dead
0 5 10 15 20 25 30 35 40 45 50
DAYS AFTER TUMOR IMPLANTATION
= Death of Single Treated Animal
= " " " Untreated "

TEXT-FIGURE 9.—Survival of tumor-bearing mice following repeated injection of 10 μg. per gm. of alpha-peltatin.

In a small number of rats bearing the carcinoma and in C3H mice bearing the adenocarcinoma, there was no appreciable increase in survival after multiple injections.

HEMATOPOIETIC AND RETICULO-ENDOTHELIAL EFFECTS

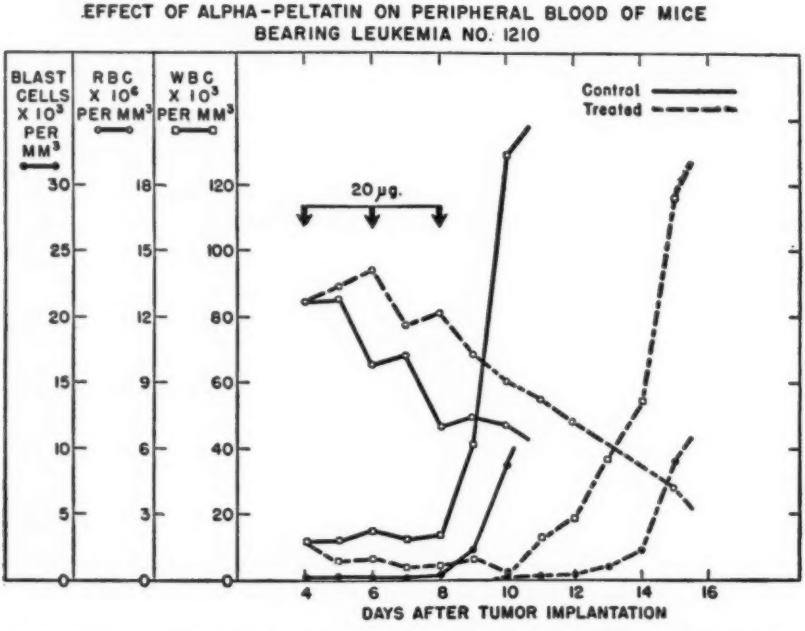
The mice with the leukemia developed a steadily rising leukocyte level, large numbers of stem cells, anemia and polychromatophilia from the 8th day after tumor transplantation until the 10th to 13th day, when most of the animals died. Treatment with multiple injections at 48- to 72-hour intervals produced a prompt leukopenia which continued during the course of the treatment. Animals treated in this manner also showed a delay in the appearance of blast forms in the peripheral blood and bone marrow, and somewhat higher erythrocyte levels (text-fig. 10 and table 4). Further details on the hematopoietic effects of the peltatins will be described separately (*30*).

TABLE 4.—*Effect of alpha-peltatin* [1] *on peripheral blood of leukemic mice*

Mice [2]	RBC	WBC	Differential count		
			Granulocytes	Agranulocytes	Blasts
	Millions/cu.mm.	*Thousands/cu.mm.*	*Percent*	*Percent*	*Percent*
Control	6.4±0.2	33.2±1.0	57	36	7
Treated	9.1±0.3	10.1±0.2	57	41	1

[1] 20 μg. per gm. was given subcutaneously on the 4th and 7th days after implantation of tumor.
[2] The data are average values for specimens obtained from four control mice and from four treated mice on the 10th day after tumor inoculation.

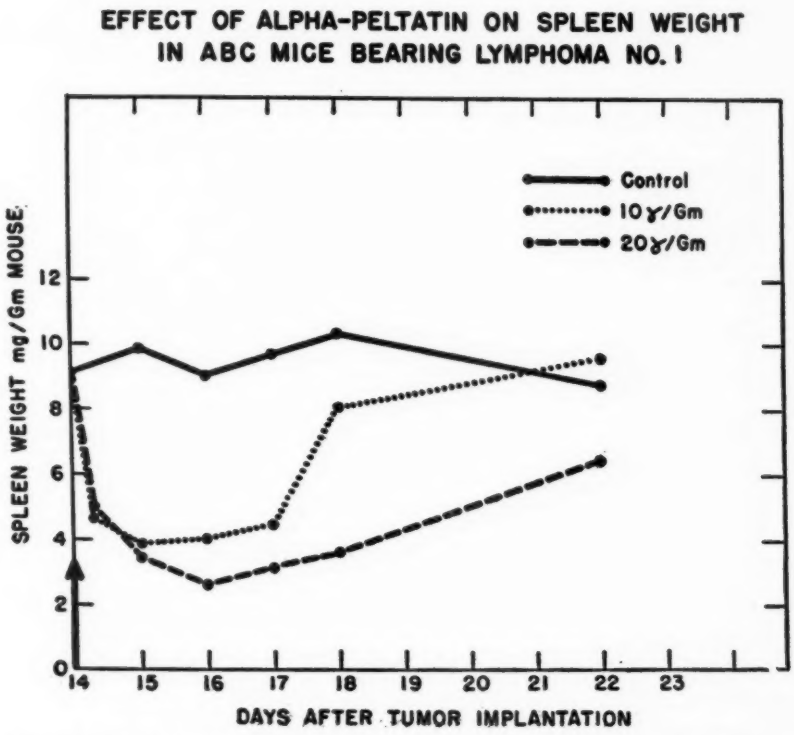
Marked shrinkage of the spleen occurred as early as 4 to 6 hours after injection of an effective dose of alpha-peltatin. This was noted in normal and in tumor-bearing mice, whether or not the spleens were infiltrated (text-fig. 11*A*, *B*). Podophyllotoxin produced a similar effect on the spleen. Thymus atrophy was observed grossly in normal animals as well as in those with tumors after a single maximum-tolerated dose and after repeated smaller doses of alpha-peltatin.



TEXT-FIGURE 10.—Each point represents the average of the findings in 4 mice. The symbols mean:

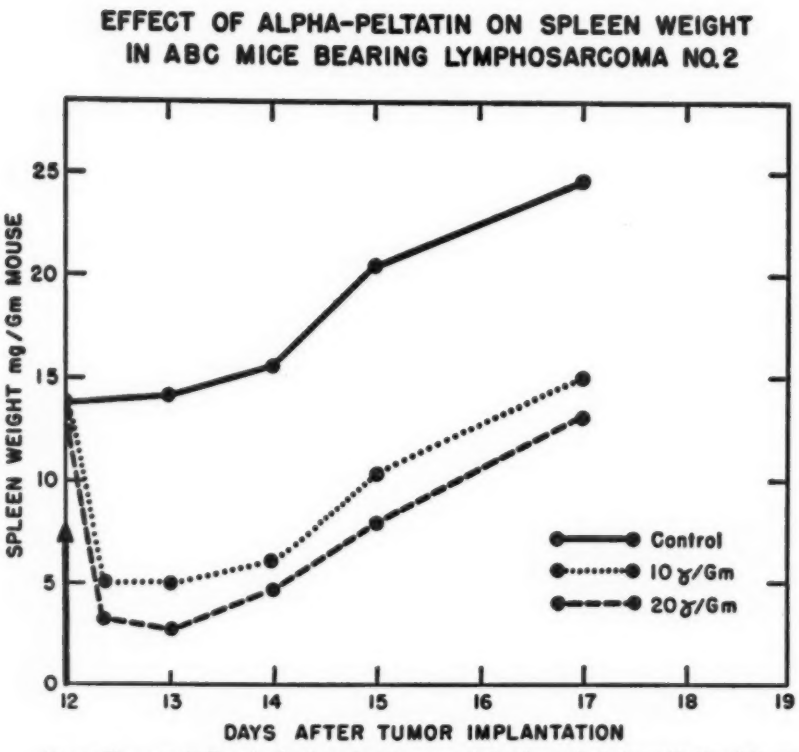
● blast (stem) cells
○ rbc
□ wbc

Note the temporary drug-induced leukopenia and the delay in the appearance of stem cells.



TEXT-FIGURE 11*A*.—A single subcutaneous injection of alpha-peltatin (10 μg. or 20 μg. per gram).

Lymphosarcoma 1. Treated on 14th day. This is a tumor localized at site of implantation; the curve for the controls shows no upward trend.



TEXT-FIGURE 11*B*.—A single subcutaneous injection of alpha-peltatin (10 μg. or 20 μg. per gram).

Lymphosarcoma 2. Treated on 12th day. This tumor rapidly infiltrates the spleen; note upward trend of the curve for the untreated mice.

DISCUSSION

In the fractionation of podophyllin, and in the isolation of the active agents from the crude drug, the response of Sarcoma 37 was employed as a guide. Hence it would not have been astonishing if the potency of the three highly active substances, *viz.*, podophyllotoxin, alpha-peltatin and beta-peltatin, had proved to be restricted to Sarcoma 37 and to closely similar tumors. It was found, however, that these substances produced extensive damage in a variety of mouse tumors such as lymphosarcoma, leukemia, carcinoma, and melanoma, and also in a rat carcinoma. The dose level required to elicit damage varied from tumor to tumor; the three types of lymphoid tumors were more readily affected than the others reported here. The relative resistance of the carcinomas and the melanoma to these agents parallels the refractoriness of these types of tumors to some chemotherapeutic agents (such as nitrogen mustards, aminopterin and urethan) and to roentgen radiation.

Complete regressions were not obtained in these experiments. By appropriate variation of the dose schedules, the growth of the tumors could be markedly retarded for a time in comparison with the control

tumors in untreated mice. In some of the experiments, the particular techniques of treatment resulted in a slight increase in survival. Effects were observed not only on the tumor mass at the injection site but also on metastases and on infiltrated bone marrow; treatment held the number of blasts in the peripheral blood down to a low level.

Following the administration of these substances to tumor-bearing mice, the animals began to lose weight. Severe damage to the tumors was seen during the first 24 hours; after another 2 days, the body weight began to increase again toward its previous level. When an equivalent weight loss was induced in tumor-bearing mice by withdrawal of food and water, the reduction in weight was not accompanied by gross or microscopic evidence of tumor damage. Mere weight loss evidently did not account for the damage induced in the tumors following administration of these substances.

These materials are considered of sufficient interest to warrant further study. The range between the maximum tolerated dose and the minimum effective dose is larger than that for colchicine and for several other compounds of interest in cancer chemotherapy. Many of the effects produced by the peltatins are similar to those obtained with colchicine; this similarity suggests a possible relationship between the mechanisms of action of these two types of compounds which will be discussed in a separate communication.

SUMMARY

Alpha-peltatin, beta-peltatin, and podophyllotoxin were injected subcutaneously into mice bearing 5 types of transplanted tumors and into rats bearing a transplanted carcinoma. The mouse tumors employed were: an acute stem-cell leukemia; a metastasizing lymphosarcoma; a local lymphosarcoma; a mammary adenocarcinoma; and a melanoma. About 1,000 animals were employed in these experiments. The results obtained were as follows:

(1) A single injection of each of these compounds induced extensive damage in all the tumor types investigated. Metastases responded like the main tumor mass.

(2) Both the minimum dose required to produce this effect and the maximum dose tolerated varied with the type of tumor and of host. The range between these dose levels was greatest for the lymphomas and least for the melanoma; it was intermediate for the carcinomas.

(3) Tumor growth was temporarily retarded in the case of the lymphomas and of the rat carcinoma.

(4) Treated animals bearing lymphatic tumors survived longer than the controls; those with the melanoma died sooner than their untreated controls. Complete regression of tumor was not obtained in any of the experiments in this study.

(5) These substances delayed the rise in the number of stem cells in the peripheral blood in mice with the leukemia.

(6) In both normal and tumor-bearing mice, treatment with these drugs caused shrinkage of spleen and thymus gland.

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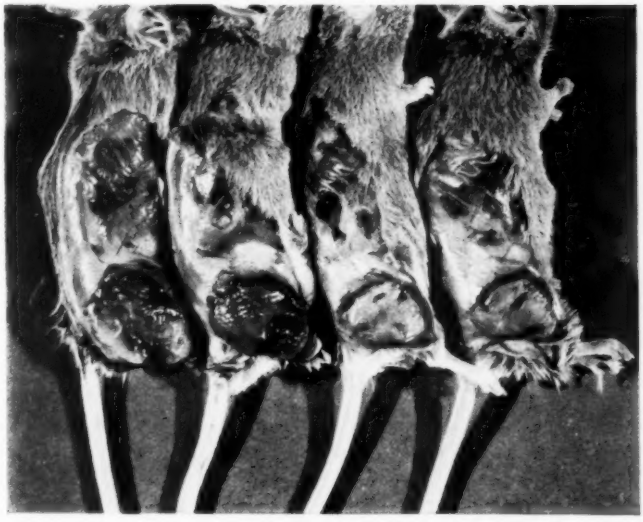
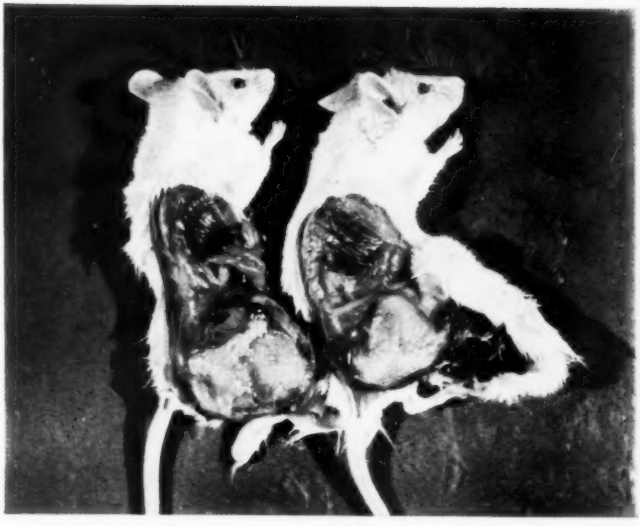
PLATE 189

FIGURE 1 (upper).—Acute stem-cell Leukemia 1210, 8-day-old transplants in dba mice, with tumors at site of implantation cut transversely. At left, two untreated control mice. At right, two mice treated 24 hours previously with a single subcutaneous injection of alpha-peltatin (20 μg. per gm.). Note the usual hemorrhage, characteristic of the untreated tumors, and the caseous necrosis in the treated ones.

FIGURE 2 (lower).—Lymphosarcoma 1, localized type, 16-day-old implants in strain A mice. At left, untreated mouse. At right, mouse treated 48 hours previously as in figure 1. Note difference in tumor size, and the caseous necrosis and hemorrhagic discoloration of the tumor bed in the treated mouse.





Greenspan *et al.*

PLATE 190

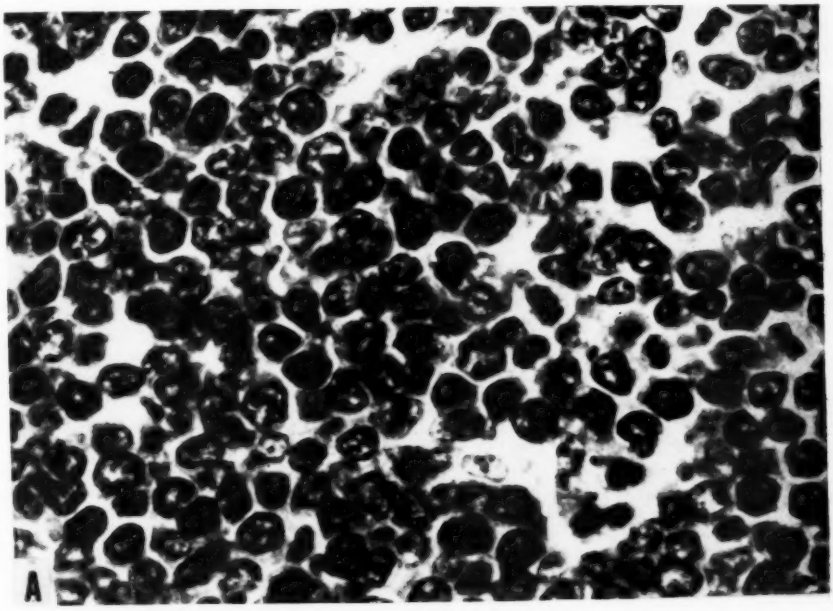
FIGURE 3 (upper).—Lymphosarcoma 1, localized type, 26-day-old implants, in strain A mice. Treatment was begun 12 days after implantation when tumors were palpable. Five doses of alpha-peltatin (10 μg. per gm.) were given subcutaneously at 72-hour-intervals. At left, two control mice. Note smaller size of tumors, and absence of hemorrhage, in the two treated mice at right.

FIGURE 4 (lower).—Rat Carcinoma 1643, 15-day-old implants, in Buffalo rats. Control animals at left. Note hemorrhagic necrosis in tumors of treated rats at right. Alpha-peltatin (5 μg. per gm.) given subcutaneously 48 hours previously.

Greenspan *et al.*

PLATE 191

FIGURE 5.—Effect of a single subcutaneous injection of alpha-peltatin upon Lymphosarcoma 1 in strain A mice bearing 15-day-old intramuscular implants. Animals killed 24 hours after the injection. *A*, untreated control tumor. *B*, tumor from mouse receiving 20 μg. per gram. Note generalized pyknosis, karyorrhexis, and chromatin dust. *C*, tumor from mouse receiving 5 μg. per gram. Note generalized pyknosis without karyorrhexis or chromatin dust. Hematoxylin and eosin stain. ×1,000.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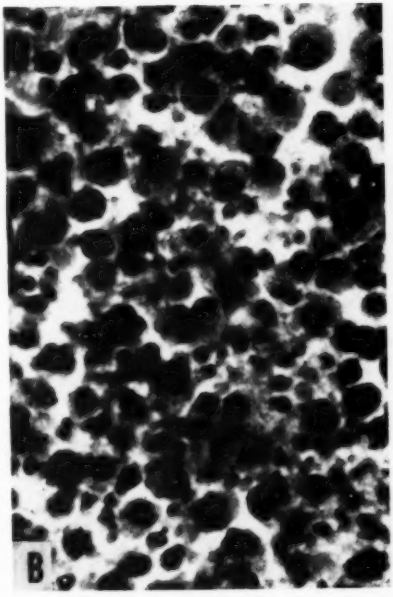
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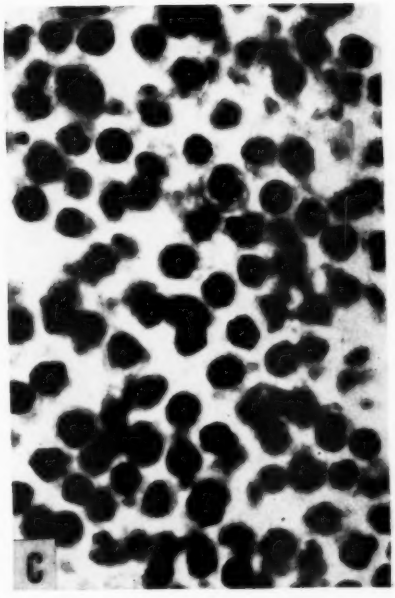
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PLATE 192

FIGURE 6.—Effect of a single subcutaneous injection of alpha-peltatin upon Lymphosarcoma 1 in strain A mice bearing 15-day-old intramuscular implants. Animals killed 24 hours after the injection. Hematoxylin and eosin stain. *A*, tumor from mouse receiving 5 μg. per gram. Section, from periphery of tumor, shows groups of apparently unaltered tumor cells around blood vessels. *B*, tumor from mouse receiving 2.5 μg. per gram, the dose inducing minimal effects in tumors. Only scattered groups of pyknotic nuclei are seen. ×1,000.

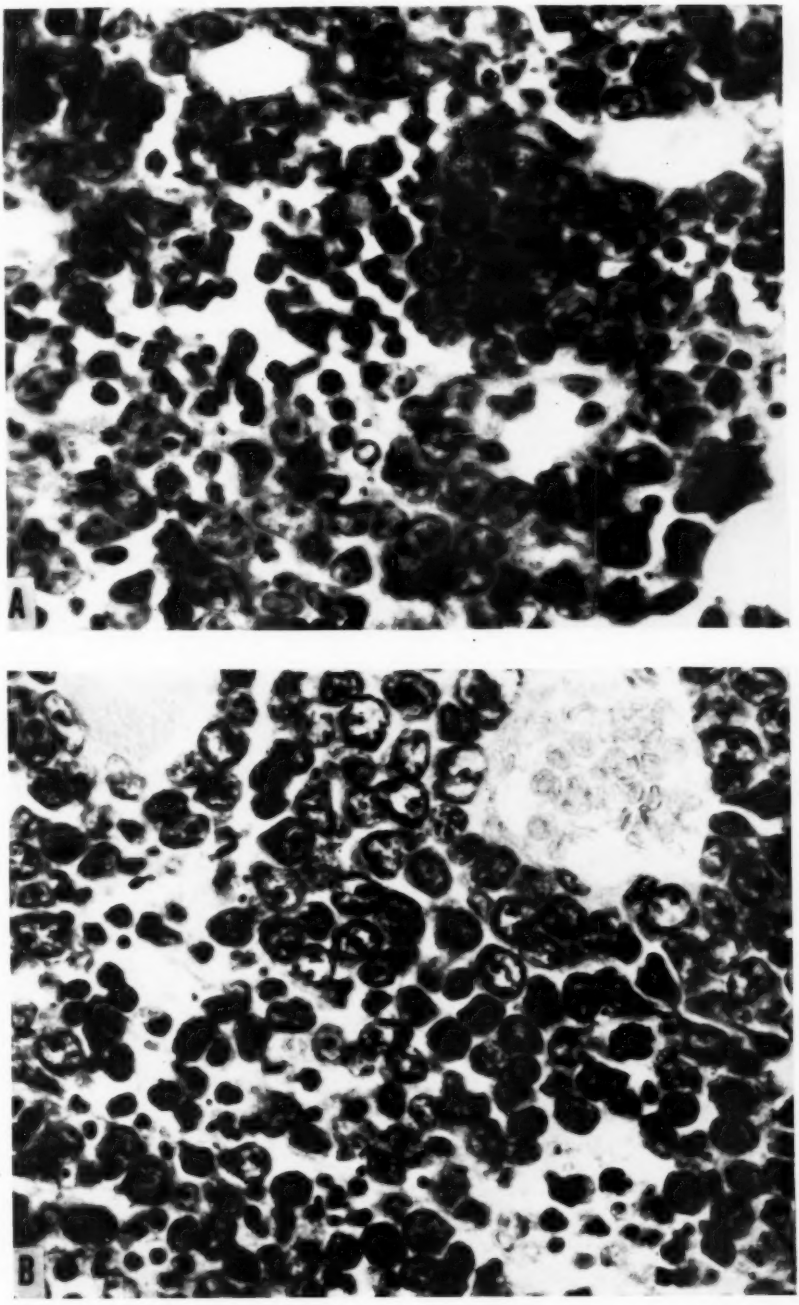
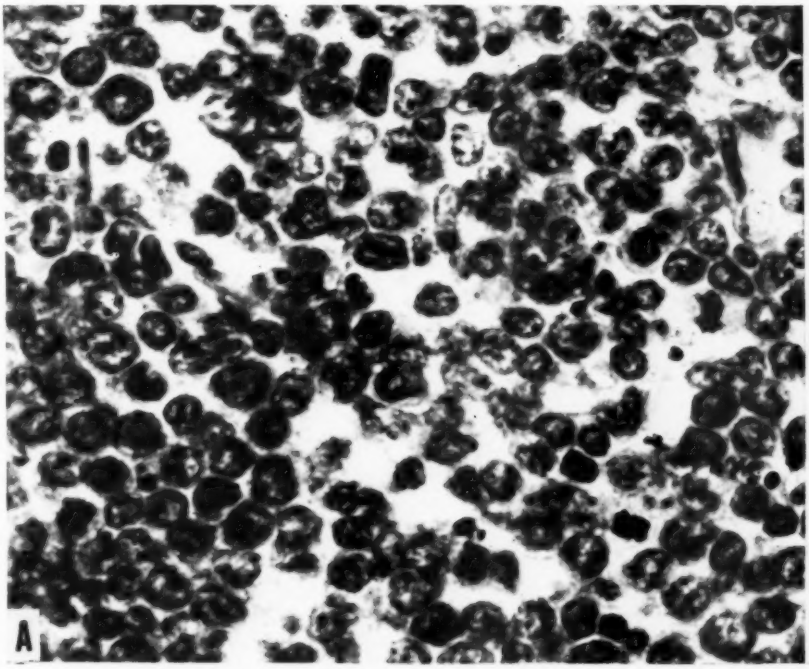

A
B

PLATE 193

FIGURE 7.—Effect of a single subcutaneous injection of alpha-peltatin upon Leukemia 1210 in dba mice bearing 8-day-old implants. *A*, untreated control tumor. *B*, tumor from mouse killed 24 hours after a relatively low dose (5 μg. per gm.) of alpha-peltatin. Note diffuse karyorrhexis, nuclear clumping, hyperchromatism, and cellular degeneration. *C*, tumor from mouse killed 48 hours after 20 μg. per gram of alpha-peltatin. Note more advanced cellular degeneration and generalized pyknosis. Hematoxylin and eosin stain. ×1,000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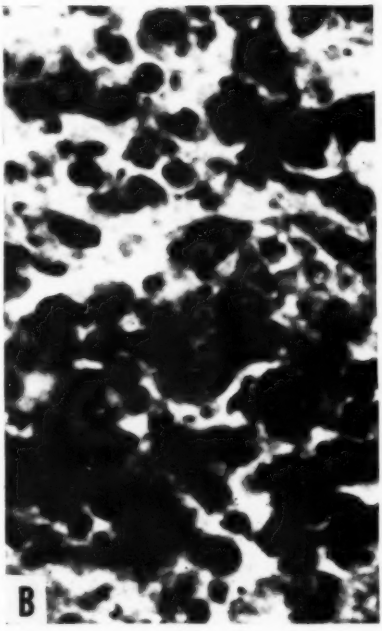
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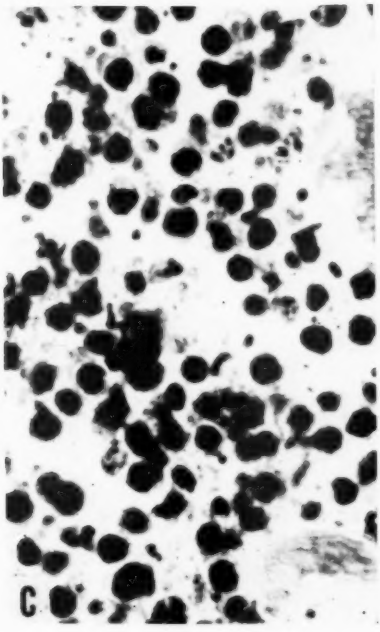
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PLATE 194

FIGURE 8.—Effect of a single subcutaneous injection of beta-peltatin or podophyllotoxin upon Leukemia 1210 in dba mice bearing 8-day-old implants. *A*, tumor from mouse killed 24 hours after 20 μg. per gram of beta-peltatin. *B*, tumor from mouse killed 24 hours after 10 μg. per gram of podophyllotoxin. Hematoxylin and eosin stain. ×1,000.

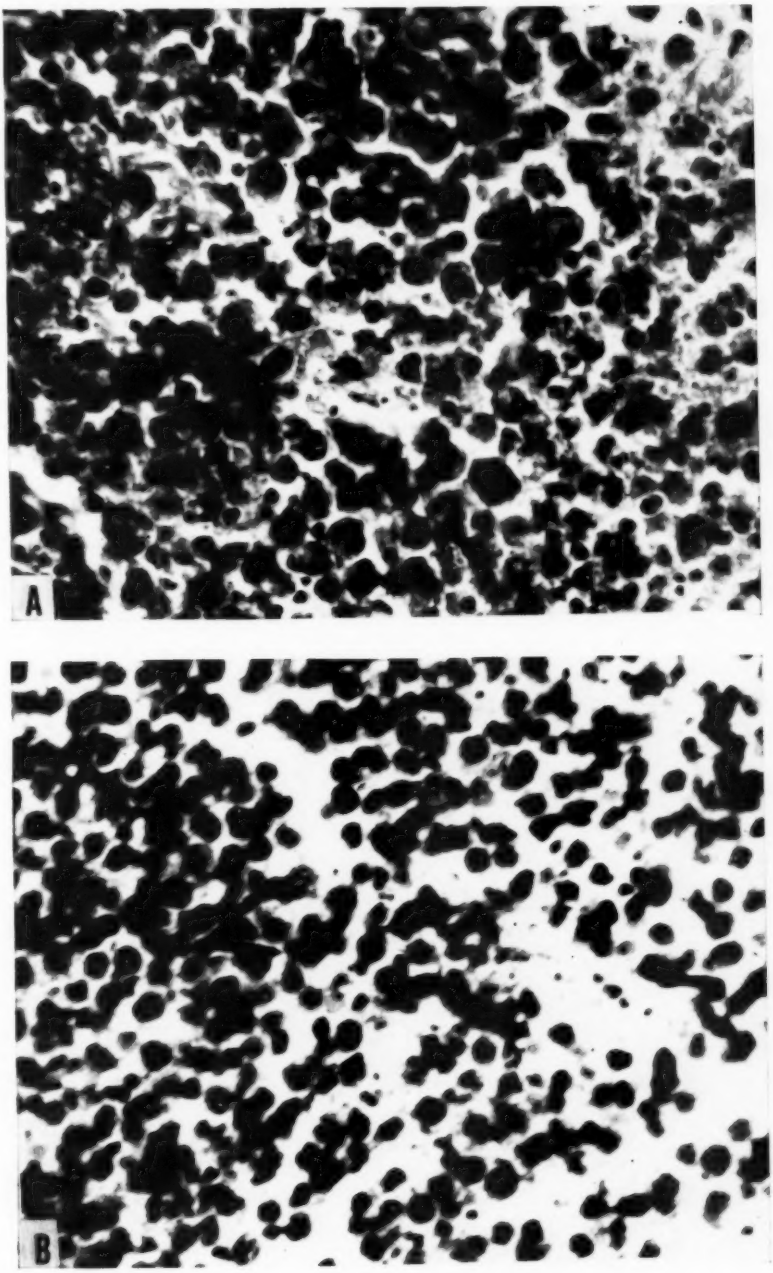
A
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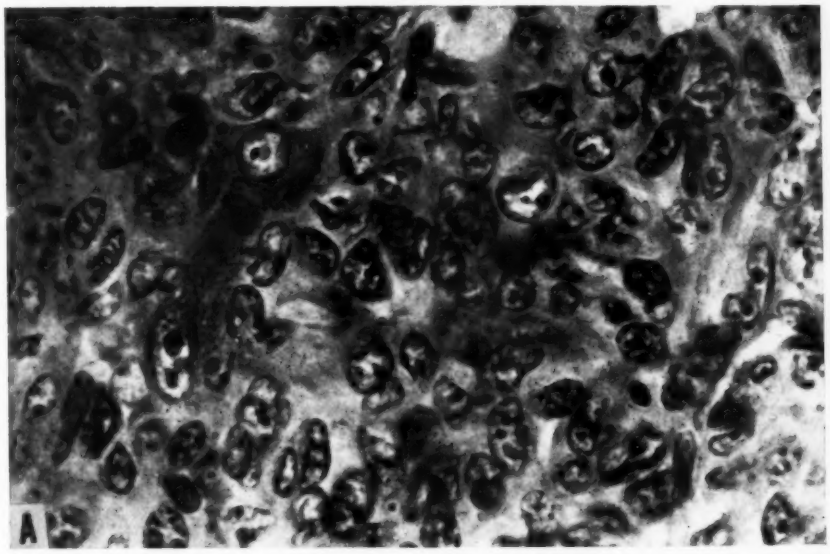
PLATE 195

FIGURE 9.—Effect of a single subcutaneous injection of alpha-peltatin upon Melanoma S–91 in dba mice bearing 21 day old intramuscular implants. *A*, untreated control tumor. Note typical polymorphic character of cells and the presence of pigment granules. *B*, tumor from mouse killed 24 hours after receiving 20 μg. per gram (a maximum tolerated dose). Note generalized pyknosis, but absence of karyorrhexis. Hematoxylin and eosin stain. ×1,000.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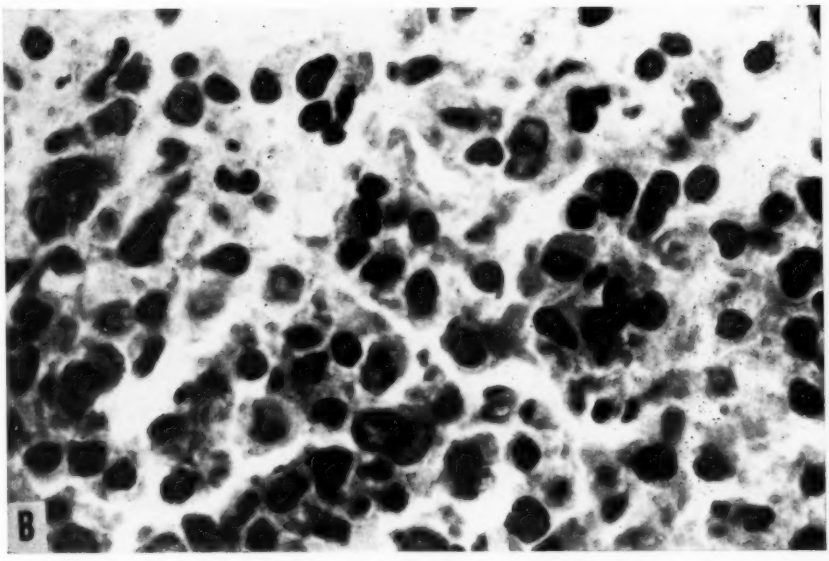
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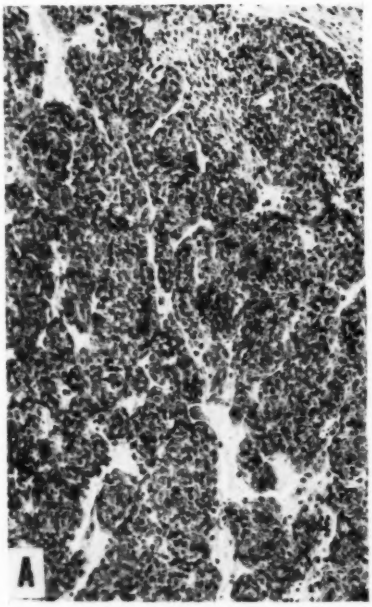
PLATE 196

FIGURE 10.—Effect of a single subcutaneous injection of 20 μg. per gram of alpha-peltatin upon mammary adenocarcinoma C3HBA in C3H mice bearing 17-day-old implants. *A*, untreated control tumor. Note characteristic well-defined acinar formation. ×155. *B*, untreated control tumor. Same as in *A*. ×1,500. *C*, tumor from treated mouse killed 24 hours after injection. Note generalized pyknosis, absence of hemorrhage, but preservation of acinar pattern. ×155. *D*, tumor from treated mouse. Same as in *C*. ×1,500.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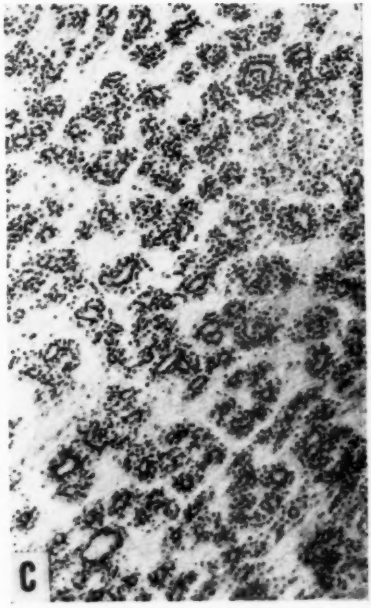
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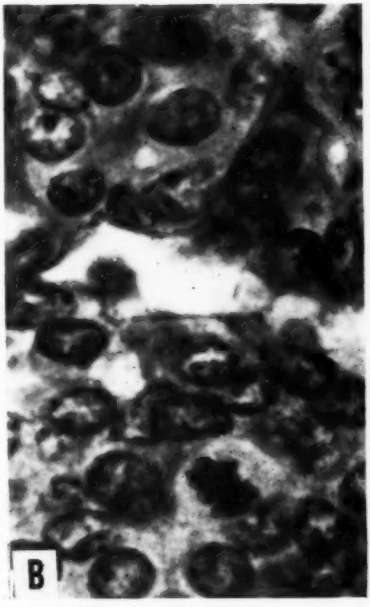
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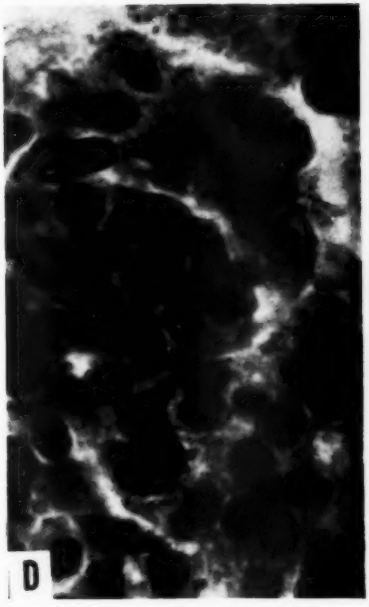
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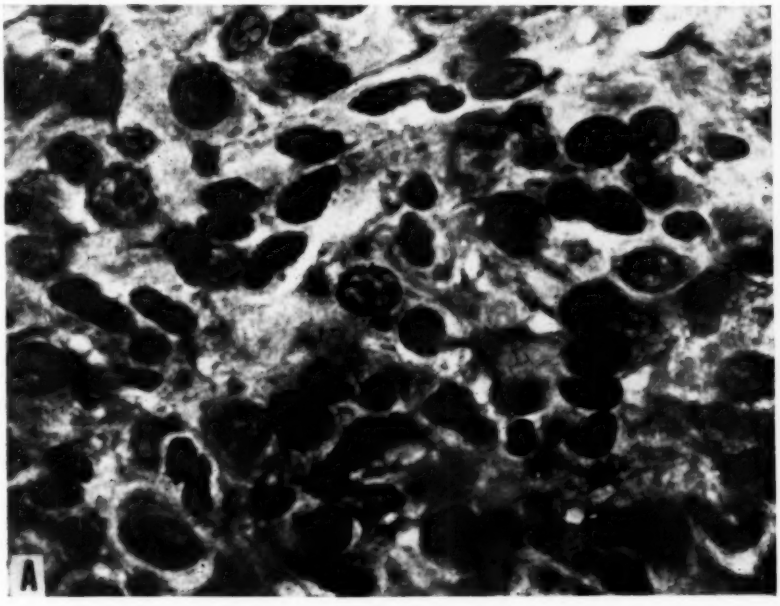
PLATE 197

FIGURE 11.—Effect of a single subcutaneous injection of 5 μg. per gram of alpha-peltatin upon Carcinoma 1643 in Buffalo rats bearing 14-day-old intramuscular implants. *A*, untreated control tumor. *B*, tumor from treated rat killed 24 hours after injection. Note generalized pyknosis. Hematoxylin and eosin stain. ×1,200.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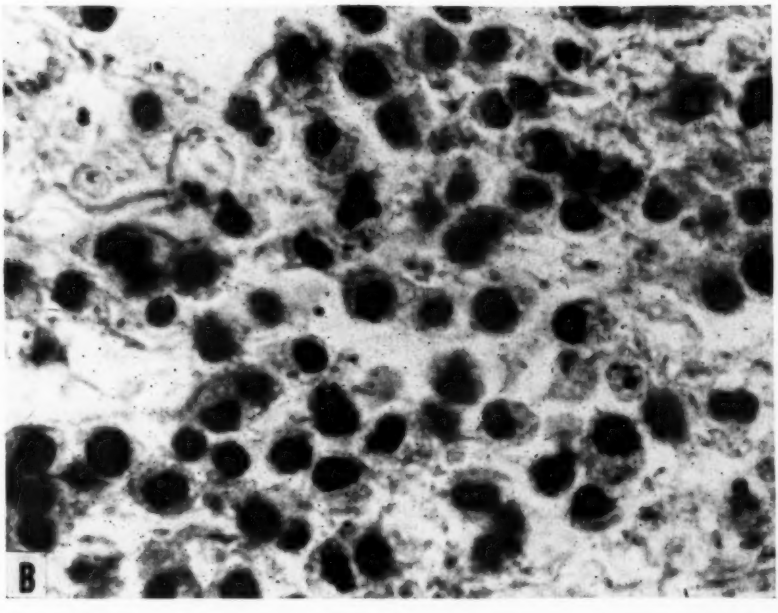
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PROCEEDINGS

OF THE FIRST MEETING OF

THE HISTOCHEMICAL SOCIETY

DEPARTMENT OF ANATOMY

UNIVERSITY OF PENNSYLVANIA SCHOOL OF MEDICINE

PHILADELPHIA

MARCH TWENTY-FOURTH AND TWENTY-FIFTH, 1950

THE HISTOCHEMICAL SOCIETY

PROCEEDINGS

Dr. George B. Wislocki, *Chairman*

BUSINESS MEETING

The first meeting of The Histochemical Society was called to order at 2 P. M., Friday, March 24, 1950, in the Department of Anatomy of the University of Pennsylvania, Philadelphia, by the Acting Secretary, Dr. Ralph D. Lillie, National Institutes of Health, Bethesda, Md. Dr. George B. Wislocki, Harvard Medical School, Boston, Mass., was elected temporary chairman, and a committee was elected from the floor to consider organizational matters and to report back to the meeting on the next day. The committee was composed of Drs. E. H. Dempsey, A. M. Seligman, R. D. Lillie, J. F. A. McManus, D. Glick, and G. B. Wislocki, who were present, and Drs. I. Gersh, A. L. Grafflin, and A. W. Pollister, who did not attend the committee meeting.

At the afternoon session on March 25, 1950, Dr. Lillie presented the report of the committee, and the meeting adopted recommendations as follows:

1. That a permanent organization be formed.
2. That the following officers be elected:

George B. Wislocki, Boston, Mass. — *President*
David Glick, Minneapolis, Minn. — *Vice-President*
Ralph D. Lillie, Bethesda, Md. — *Secretary*
Edward H. Dempsey, Boston, Mass.* — *Treasurer*

Councillors

One Year

O. Lowry, St. Louis, Mo. — George Gomori, Chicago, Ill.

Two Years

I. Gersh, Chicago, Ill. — Stuart Mudd, Philadelphia, Pa.

Three Years

Arnold B. Seligman, Boston, Mass.
J. F. A. McManus, Birmingham, Ala.**

Four Years

C. P. Leblond, Montreal, Que.
J. W. Bartholemew, Los Angeles, Calif.

3. That the Council draft a constitution to be submitted to the Society next year.
4. That membership applications be submitted to a membership committee. That their recommendations be circulated to the council for mail vote for election. That reasonably high qualifications for membership be set.
5. That annual dues of $3.00 be levied on members, and that the registration fee for meetings be continued.

The secretary reported receipts of $212.00 in registration fees, and requested authorization for repayment of postage and miscellaneous expenses prior to this meeting, $18.00 in amount; for payment of the bill for badges, $18.00 in amount; and of the bill for transcription of the discussions, estimated as probably not over $50.00; and requested an appropriation of $25.00 for postage and stationery for the secretary. This authorization was specifically included in a blanket ratification of proposals 3 to 5 above.

*Present address: Washington University, St. Louis, Mo.
**Present address: University of Virginia, Charlottesville, Va.

After a short recess, while the scientific program continued, with Dr. Glick presiding, Dr. Wislocki appointed the following committees:

1. For the nomination of officers and two new councillors for 1951: Dr. A. L. Grafflin, Baltimore, Md.; Dr. Roger D. Baker, Birmingham, Ala.; Dr. E. D. DeLamater, Philadelphia, Pa.; and Dr. L. E. Thomas, Columbia, Mo.
2. For publication of the transactions: Dr. Stuart Mudd, Philadelphia, Pa.; Dr. E. W. Dempsey, Boston, Mass., Dr. O. Lowry, St. Louis, Mo.; and Dr. R. D. Lillie, Bethesda, Md., who will edit them.
3. Membership: Dr. O. Lowry and Dr. R. D. Lillie.
4. The Council is to draft the constitution.

[*Editor's Note.*] The council has since voted to hold the next annual meeting in Detroit, March 19–20, 1951.

INDEX OF AUTHORS

Scientific Proceedings of the Histochemical Society

	Page
Arzac, J. P	1341
Atkinson, W. B	1372
Ayres, W. W	1380
Baker, R. D	1378
Blank, H	1372
Bunting, H	1368
Burgos, M. H	1377
Cason, J. E	1343
Chu, C. H. U	1344
Cleveland, F. P	1379
Coons, A. H	1344
Coriell, L. L	1372
Crouse, H. V	1372
Davenport, H. A	1380
Deane, H. W	1344
DeLamater, E. D	1355, 1375
DeLustig, E. S	1371
Drabkin, D. L	1357
Elftman, H	1342
Emmel, V. M	1365
Firminger, H. I	1350
Flax, M	1349
Flesch, P	1370
Follis, R. H	1370
Friedenwald, J. S	1364, 1367, 1379
Gall, E. A	1379
Glick, David	1347
Goetsch, J. B	1364
Gomori, G	1371
Greep, R. O	1373
Gross, J	1353
Hamilton, L	1374
Hayes, E. R	1377
Himes, M	1349
Hoover, C. R	1375
Kalfayan, B	1380
Kaplan, M. H	1344
Kent, S	1378
Kidd, J. G	1380
Koelle, E. S	1364
Koelle, G. B	1364
Koenig, H	1346
Kurnick, N. B	1345, 1373
Leduc, E. H	1348
Leuchtenberger, C	1349
Lillie, R. D	1344
Liu, C	1375

	Page
MacCardle, R. C	1372
Maengwyn-Davies, G	1379
Mancini, R. E	1371, 1376, 1377, 1378, 1379
McManus, J. F. A	1343
Mellors, R. C	1358
Mescon, H	1370
Montagna, W	1376
Moses, M. J	1345
Mowry, R. W	1373
Mudd, S	1354, 1355
Nadel, E. M	1366
Pollister, A. W	1349
Popper, H	1374
Porter, R. W	1380
Richfield, D	1379
Robinow, C. F	1374
Schiff, L	1379
Scott, T. F. M	1372
Smith, A. G	1354, 1355
Smith, C	1376
Sparrow, A. H	1345
Steele, R	1345
Steinitz, L. M	1346
Stowell, R. E	1361, 1367
Stuart, E. G	1375
Sulkin, N. H	1346
Szanto, P. B	1374
Thomas, L. E	1346, 1375
Tsuboi, K. K	1367
Villamil, M. F	1379
Wilson, J. W	1348
Wislocki, G. B	1341
Wright, P	1372
Wurzbacher, W	1364
Yokoyama, H. O	1367
Zorzoli, A	1366

PAPERS READ

Saliva-Insoluble Glycoproteins, Stained by the Periodic Acid Schiff Procedure, in the Placentas of Pig, Cat, Mouse, Rat, and Man. G. B. WISLOCKI, *Harvard Medical School, Boston, Mass.*

Abstract. In *pigs' placentas*, a positive reaction is given by secretion droplets in the uterine glandular and surface epithelia; this secretion reaches the uterine lumen and appears to be absorbed by the columnar epithelium of the chorionic fossae and areolae. Unlike epithelial mucus (acid mucopolysaccharides), this "serous" secretion is neither metachromatic nor strongly basophilic upon staining with toluidin blue. In the chorionic lamellae of *cats' placentas*, strongly positive material, which appears to be decidual in origin, intervenes between the fetal syncytium and the maternal blood vessels. In the paraplacental "brown border," material in the epithelium and lumen of the uterus and in the chorionic epithelium reacts positively. In *rodents' placentas*, the uterine epithelium, the secretion present in the uterine and vitelline cavities, Reichert's membrane, and the epithelium of the parietal and visceral layers of the vitelline membrane react strongly. In the animals cited a glycoprotein, differing from acid mucopolysaccharides, is secreted by the uterine epithelium and is absorbed by special regions of the placenta. In *human placentas*, the uterine glandular epithelium and its secretion are periodic acid-Schiff positive, but the secretion is also metachromatic. The decidual ground substance is positive and strongly metachromatic (acid mucopolysaccharide). The "decidual" cells are negative, but scattered small cells of the decidua react positively. The marginal cytoplasm and brush border of the syncytial trophoblast are positive although not metachromatic; the Langhans cells are negative, whereas the peripheral cytotrophoblast is faintly positive (chorionic gonadotrophin).

Discussion

DR. R. D. LILLIE (*Bethesda, Md.*): I might remark that the difference in species of the metachromasia and of the periodic Schiff reaction finds its parallel in the gastric mucin, which is strongly metachromatic, for instance, in the dog, and fails to stain utterly with the metachromatic dyes in several other species, but gives a strong periodic Schiff reaction in all of them.

DR. ARTHUR HASS (*Baltimore, Md.*): I would like to ask Dr. Wislocki whether the materials he refers to as metachromatic are metachromatic after dehydration, in balsam, or whether that refers to observations on wet material?

DR. WISLOCKI: The metachromasia observed here is that which follows the use of basic lead acetate as a fixative, paraffin embedding, and staining in a one-half percent solution of toluidin blue.

A Simple Histochemical Reaction for Aldehydes. J. P. ARZAC, *Hospital Español, Mexico, D. F.*

Abstract. A study has been made on the possibility of replacing leucofuchsin by colored basic fuchsin for the histochemical demonstration of aldehydes.

Several tissues from mammals and various pertinent fixatives were used. Two methods were utilized to free aldehydes: (a) oxidation with several combinations that yield periodic acid, besides chromic acid and potassium permanganate, to liberate aldehydes from carbohydrates; (b) hydrochloric acid hydrolysis to unmask aldehydes from thymonucleic acid. The effects of sulphurous acid in relation to the histochemistry of the reaction, of acidity, and of substances of mordanting action were also studied, as well as the effect of blocking the radicals assumed to take active part

in the reaction and the acid-fastness of the substances revealed with both techniques.

It was found that the colored form and not necessarily the leucoform of basic fuchsin can be used histochemically in demonstrating aldehydes. A technique, which is described, using the colored form is considerably simpler, shorter, more economical and as reliable as its equivalent using leucofuchsin in mammal tissues.

The chemistry assumed to be in play is the following: (a) triphenylmethane amino groups and aldehyde radicals are unstably attached to each other; (b) sulphurous radicals modify the quinoid linkage and determine the formation of an insoluble compound; (c) resonance induced by the addition of acid regenerates the quinoid form in the ring containing the linked amino-aldehyde groups.

Discussion

DR. R. D. LILLIE (*Bethesda, Md.*): On brief trial of the method we found identical demonstration of glycogen, starch, and cellulose by the Arzac and our own HIO_4 Schiff techniques.

Gastric and intestinal mucins were colored by both procedures but the color was lighter by the Arzac method.

Collagen remained unstained and reticulum was inconclusively demonstrated in our few preparations. Brush borders of renal epithelium remained essentially uncolored.

The alteration from the effect seen in our usual preparations is similar to but more pronounced than that introduced by the use of the Hotchkiss acidified thiosulfate rinse, and closely resembles that produced by interposition of a 10- to 20-minute bath of M/20 acid sodium sulfite after the periodic oxidation and before the Schiff reagent bath.

The Application of Oxidation Reactions to the Study of Spermatogenesis. H. ELFTMAN, *College of Physicians and Surgeons, Columbia University, New York, N. Y.*

Abstract. Spermatogenesis in the mouse and in man has been investigated by means of oxidation by periodic acid, silver diammine, Aoyama's silver method, osmium tetroxide, and ferric chloride-ferricyanide. For general study, fixation in Bouin was followed by HCl hydrolysis, periodic acid oxidation, leucofuchsin and counterstaining in fast green. Periodic acid oxidizes constituents of the acrosome, idiozome, cytoplasmic granules in the germ cells and Sertoli cells, and mitotic spindles, in addition to delineating the cell boundaries and the reticular fibers and collagen. The effect of periodic acid on the Golgi apparatus was compared with the results achieved by standard methods by applying periodic acid, followed by leucofuchsin, to alternate sections of Aoyama silver and of osmic preparations. Silver diammine is reduced by the products of periodic acid oxidation but it is also reduced directly by the acrosome material. Aoyama's silver method visualizes not only the Golgi apparatus but also the cytoplasmic processes of the Sertoli cells. The reactions described, combined with others, provide information concerning the chemical constitution of the cellular constituents.

Discussion

DR. G. B. WISLOCKI (*Boston, Mass.*): Differences in the histochemistry of sperm will be interesting, particularly in reference to the different places where they can be obtained, namely, from the testis, epididymis and vas deferens, as well as from different parts of the female reproductive tract after coitus.

I published several figures in 1948 of sperm in deer testes as revealed by various histochemical reactions. The acrosomes were intensely stained in spermatids by the periodic acid-Schiff reaction in deparaffinized sections.

More recently I have attempted to stain smears of human seminal fluid containing sperm and seminal plasma. These smears, made on slides, have been stained by various histochemical methods including the periodic acid-Schiff procedure. Although I observed intense staining of the acrosomic material in the testis of deer, I have failed to observe any periodic acid-Schiff positive material in ejaculated human sperm. On

the other hand, precipitated human seminal plasma is strongly periodic acid-Schiff positive.

MR. Y. CLERMONT (*Montreal, Quebec*): Dr. Elftman would probably be interested to know that, with the help of the periodic acid-Schiff reaction, we have been able to follow the details of the spermiogenesis in the testis of rat, guinea pig, and some other species.

This was made possible by the fact mentioned by Dr. Elftman that the proacrosomic granules found in the Golgi area of the spermatid react to the periodic acid-Schiff technique. Those granules transform into the head cap of spermatozoa and a strongly reactive acrosome-like structure. The reactivity of those structures gradually diminishes as the spermatozoa are released from the Sertoli syncytium.

Incidentally, in the cytoplasm of the Sertoli cells, some periodic acid-Schiff-reactive granules may be observed. They are modified according to a cycle corresponding to the spermatogenic wave.

DR. LOTTI STEINITZ (*Columbia, Mo.*): I should like to present some supporting evidence to Dr. Elftman's statement that Bouin fixation causes a certain amount of acid hydrolysis of D. N. A. Mr. Charles R. Hoover of the Biochemistry Department of the University of Missouri, who was to present a paper here on his microspectrophotometric studies, worked with preparations of thymus nucleohistone. He observed that this material, embedded in agar, fixed and carried through the usual histological procedures, gave a weak Feulgen stain after Bouin's fixation without any acid hydrolysis. I myself have noted in histological preparations that after Bouin fixation the color produced by the Feulgen reaction is less intense and more bluish than that obtained after Carnoy fixation (both with short acid hydrolysis). Mr. Hoover has confirmed this quantitatively with his microspectrophotometer.

DR. ELFTMAN: The fact that three laboratories have independently obtained significant results by the application of the periodic acid-leucofuchsin method to the study of spermatogenesis confirms the usefulness of this approach. The variability found in the staining of the mature spermatozoa of different animals by this method may eventually prove highly significant in the interpretation of the physiological maturation of the sperm. We do not feel that it is justifiable as yet to conclude that the reaction is necessarily due to a polysaccharide.

The determination of arginine was made by Baker's method of applying the Sakaguchi reaction, with a slight modification which we have introduced in the final mounting of the preparation.

Carbohydrate Histochemistry Studied by Acetylation Techniques. I. Periodic Acid Methods. J. F. A. MCMANUS and J. E. CASON, *Medical College, University of Alabama, Birmingham, Ala.*

Abstract. An acetic anhydride pyridine mixture acetylates hydroxyl groups in tissue sections. The acetyl groups can be removed from tissue sections by weak alkali.

Sections acetylated by acetic anhydride pyridine no longer produce aldehydes after oxidation by periodic acid. Acetylated sections subjected to weak alkali regain the ability to produce aldehydes after oxidation by periodic acid.

Acetylation of tissue sections and the removal of acetyl groups by weak alkali can be used as histochemical confirmation of the carbohydrate nature of material coloring with Schiff's reagent after periodic acid.

Acetylation of tissue sections shows the presence of free hydroxyl groups in the tissues. Other methods of staining—silver impregnation, fat and metachromatic staining—may be studied by acetylation to question their dependence on free hydroxyl groups.

Discussion

DR. ARNOLD M. SELIGMAN (*Boston, Mass.*): I think the decision whether these experiments prove that 1,2-glycols are indeed involved in the periodic acid reaction hinges on whether or not pyridine and acetic anhydride will acetylate only 1,2-glycols.

It is my impression that these reagents will acetylate nearly everything in sight—alcohols, amines, 1,2-glycols, or any other glycols. Don't you think that is true?

DR. MCMANUS: There are several points that you brought up, Dr. Seligman. I think that first of all it will acetylate amines as well as glycols. The weak alkali will not saponify the acetylamines in this method, but only return the hydroxyl groups to the glycol. Step 3, then, is important.

(The discussion during the rest of the session was such that it was impossible for the reporter to hear it well enough to make an intelligible transcript.)

Further Exploration of the HIO_4-Schiff Reaction With Remarks on Its Significance. R. D. LILLIE, *National Institutes of Health, Bethesda, Md.*

Abstract. Recent literature indicates that many writers are regarding the HIO_4-Schiff reaction as practically specific for mucopolysaccharides and mucoproteins provided glycogen and galactolipins are appropriately excluded.

Author reports on positive HIO_4-Schiff reactions of salivary zymogen granules of several species, pancreatic zymogen and Paneth cell granules of some species and not of others, of adrenal chromaffin, and of adrenal "lipofuscin" and intestinal "melanin" or "pseudomelanin" pigments and negative reactions of cutaneous melanin, most mast cell granules, peptic zymogen granules, and splenic hemosiderin.

Author discusses positive HIO_4-Schiff reactions of these and other nonmucoid substances and suggests a return to the original interpretation of the Malaprade reaction as indicating adjacent glycol or hydroxyamino groupings.

A Histochemical Study of Staining the Axis Cylinder With Fuchsin-Sulfurous Acid (Schiff's Reagent).[1] C. H. U. CHU,[2] *Sloan-Kettering Institute, New York, N. Y.*

Abstract. Axis cylinder in fresh tissue is stained with fuchsin-sulfurous acid (Schiff's reagent) without preliminary fixation or hydrolysis. This property no longer exists when the tissue is treated with alcohol before or after staining. Ethyl alcohol extracts from nerve some substance that changes the leucofuchsin to a violet-colored compound.

Extracts of mouse brain with aldehyde-free alcohol were subjected to chemical tests for (1) bromine, (2) aldehyde, and (3) unsaturated fatty acid. Evidence is presented to demonstrate that the substance responsible for the typical color-change is an unsaturated fatty acid derived from lecithin.

The Histochemical Detection of Antigens. A. H. COONS, M. H. KAPLAN, and HELEN W. DEANE, *Harvard Medical School, Boston, Mass.*

Abstract. The precipitation of antibody by specific antigen has been employed as a histochemical reaction for the localization of antigenic material in tissue sections. The essential reagent is a conjugate of antibody protein with fluorescein isocyanate. When such a solution is applied to a tissue section a microprecipitate forms over the sites containing specific antigen. This precipitate can be visualized under the fluorescence microscope. Precautions must of course be taken to ensure that the antigen is not inactivated or dissolved during the preparation of the sections, and that the observed fluorescence is immunologically specific.

Our usual method of preparing sections is that of Linderstrøm-Lang and Mogensen, which consists of cutting frozen sections of unfixed tissue in a refrigerated cabinet. Polysaccharide antigens were studied in tissues fixed in a solution of picric acid,

[1] This investigation was aided by a grant (C1180) from the United States Public Health Service.

[2] Postdoctorate Fellow of the National Cancer Institute of the United States Public Health Service.

alcohol, and formalin, and embedded in paraffin. Controls of specificity were: the absence of precipitate on sections from normal animals, and inhibition of the reaction by prior treatment of a parallel section with unlabeled antibody.

The method has been successful in the localization of injected pneumococcal and Friedländer bacillus polysaccharides, of rickettsiae, of mumps virus, and of plasma proteins from various species.

Quantitative Determination of Nucleic Acids During Meiosis in *Trillium*.[1] M. J. Moses, R. Steele, and A. H. Sparrow, *Brookhaven National Laboratory, Upton, N. Y.*

Abstract. Quantitative determinations of pentose and desoxypentose nucleic acids (PNA and DNA) have been made on the anther contents (pollen mother cells) of *Trillium erectum* at various stages of meiosis. Methods of quantitative microchemical and cytochemical analysis were employed and will be discussed. The data (amounts of DNA per cell) as obtained by both methods will be presented and compared. Similarities and differences between them will be discussed with respect to nuclear changes during division.

Methyl Green-Pyronin: Basis of Selective Staining and Histochemical Application. N. B. Kurnick, *Tulane University, New Orleans, La.*

Abstract. The peculiar selectivity of these two basic dye-stuffs for two closely related acid substrates, desoxyribonucleic acid and ribonucleic acid, appears to depend upon the state of polymerization of the substrate. Qualitative and quantitative studies were performed which demonstrated that methyl green stains highly polymerized desoxyribonucleic acid, and not depolymerized desoxyribonucleic or ribonucleic acid (found in tissues only as a low polymer). Pyronin, on the other hand, stains preferentially depolymerized desoxyribonucleic acid and ribonucleic acid, but not polymerized desoxyribonucleic acid. Histone and lanthanum compete with methyl green for the polymerized desoxyribonucleic acid.

Quantitative studies demonstrated that methyl green (C. I. #684) stains polymerized desoxyribonucleic acid in the ratio of ten phosphoric groups per dye molecule. (For ethyl green, also labeled methyl green C. I. #685, the value is 13P per dye molecule.) This stoichiometry was confirmed by precipitation experiments with analyses of the desoxyribonucleic acid—methyl green precipitate, of the remaining supernate, and by dialysis. Furthermore, it was demonstrated that free methyl green fades rapidly outside the pH range 3.5–5.0. The desoxyribonucleic acid-methyl green complex, however, is stable at higher pH's also. This permitted the confirmation of the stoichiometry by adjusting the pH of desoxyribonucleic acid-methyl green mixtures, in which the latter was in excess to pH 7.5, and allowing the uncombined methyl green to fade.

The constant stoichiometry of the reaction permits the application of methyl green in a quantitative histochemical method. A successful method was devised which depends upon the preparation of homogeneous nuclei with sucrose solution and formalin fixation, removal of histone, staining at pH 4.1, and decolorizing with a buffer at this pH. It is important to avoid depolymerization of the desoxyribonucleic acid, which may occur inadvertently during embedding. A temperature of 60° C for twelve minutes has been shown to depolymerize desoxyribonucleic acid.

[1] Research carried out at Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

The Use of Perchloric Acid for the Histochemical Demonstration of Ribonucleic Acid in Vertebrate Tissues.[1] N. H. SULKIN, *St. Louis University School of Medicine, St. Louis, Mo.*

Abstract. Using photocolorimetric estimation, Ogur and Rosen (1949) present data which indicate that cold perchloric acid extracts pentose nucleic acid from plant tissue residues. Seschachar and Flick (1949) report that PNA is extracted from *Chilodonella* by this treatment. This method has been adapted for use with vertebrate tissues.

Treatment of deparaffinized sections on slides with cold perchloric acid had no significant effect. The following techniques were devised.

A. Tissues from rats and cats (liver, autonomic ganglia, sensory ganglia, pancreas, and thyroid and salivary glands) were fixed for 8–15 hours with Zenker's fluid and were washed in running water for 2 hours. One-half of each block of tissue was placed in 10 percent perchloric acid for 18 hours at 5° C. The other half of each block served as controls. The tissues were again washed for 2 hours, dehydrated, embedded and sectioned in the usual manner, and stained with Mallory's phloxine-methylene blue.

B. Tissues, similar to those in A, were fixed and washed as above. Frozen sections, at 15 microns, were placed in 10 percent perchloric acid for 18 hours, rinsed in water, stained in 1 percent toluidin blue for 5 minutes, counterstained in 0.5 percent alcoholic eosin, and mounted in glycerin.

In both methods, the results were identical to those in which ribonuclease is used as the digestive agent.

Differential Extraction of Nucleic Acids From Mammalian Nerve Cells With Perchloric Acid. H. KOENIG, *Chicago Medical School, Chicago, Ill.*

Abstract. Ogur *et al.* (Fed. Proc. 1949, 8: 234; Science, 1949, 110: 472) have employed perchloric acid for the selective extraction of ribose nucleic acids and desoxyribose nucleic acids from plant tissues. This is a preliminary report of work underway to apply the foregoing procedure to mammalian nerve cells as a cytochemical technique. Spinal cords of cats and guinea pigs were fixed by vascular perfusion with 10 percent formalin, cut 10 μ thick and mounted on slides. After deparaffinizing and passage through water, slides were placed in 10 percent perchloric acid for varying periods of time and at several temperatures along with control slides in water. One series of experimental and control slides was stained with thionin at pH 3.24, another with hematoxylin and eosin.

Nerve cells treated with 10 percent perchloric acid at 19.5° C showed incipient reduction in basophilia of cytoplasm and nucleus at 45 minutes. By 6 hours only traces of basophilia persisted, disappearing by or before 24 hours. Nuclear basophilia of glial, endothelial and ependymal cells progressively diminished though not quite so rapidly. Sections treated with 10 percent perchloric acid at 25° C showed no cytoplasmic basophilia in nerve cells at two hours, and possibly earlier, although glial and other nuclei stained well. Sections stained with hematoxylin and eosin showed no appreciable alterations even when no trace of basophilia remained. This suggests that nucleic acids are removed without influencing cellular morphology and protein structure. This point is being further studied, and work is under way to remove selectively ribonucleic acid at lower temperatures.

Histochemical Study of Epidermal Carcinogenesis in the Mouse. LOTTI M. STEINITZ and L. E. THOMAS, *University of Missouri, Columbia, Mo.*

Abstract. Changes in cellular protein during epidermal carcinogenesis were studied by means of the arginine, Millon, and diazo histochemical methods. By using sections

[1] This investigation was supported by a research grant from the National Heart Institute, U. S. Public Health Service.

of agar blocks containing protein we have ascertained that differences in protein concentration of about 15 percent can be detected visually by comparison of the color intensities. In primary squamous-cell carcinomas of mice (induced by painting the skin of the back with methylcholanthrene) the cytoplasmic protein-bound arginine, as determined by the arginine histochemical method, is greatly increased over that present in normal epidermis. This increase appears to be greater than can be accounted for by the frequently coincident increase in total proteins, as determined by the Millon and diazo histochemical methods. It is therefore highly probable that the ratio of arginine-rich protein, *i. e.*, protein of the histone type, to the total protein is greater in the primary squamous-cell carcinoma than in normal epidermis or epidermis made hyperplastic by painting with methylcholanthrene. The implications of these observations and the effect of various extractions preceding the histochemical procedures will be discussed.

The Use of X-Ray Absorption in Cytochemistry. DAVID GLICK, *University of Minnesota, Minneapolis, Minn.*

Abstract. From the pioneering work of Engström at the Karolinska Institute, Stockholm, it is now possible to apply the absorption of monochromatic X-radiation to the quantitative determination of all elements of biological importance, other than hydrogen, in morphological units down to one micron in diameter. A recent modification has extended the usefulness of this technique to the measurement of dry weight and certain organic substances in a like fashion. Principles, apparatus, and technique will be briefly discussed, and applications to elementary and mass analyses in a wide variety of tissues and cells will be reviewed to demonstrate the scope and type of problems that can be studied in this manner.

Discussion

DR. STUART MUDD (*Philadelphia, Pa.*): I would like to ask Dr. Glick if he finds any evidence in the staining properties or other processing properties of changes made by the radiation?

DR. GLICK: We observed no effect on the staining. This work was on frozen-dried material and the measurements were carried out in high vacuum. Under those conditions, the radiation does not affect subsequent staining. But remember that the x-ray radiation is quite weak, longer wavelengths being used.

DR. JOHN BUCK (*Bethesda, Md.*): You confused me a little by the stated magnification of the human skin picture. You meant that of the lantern slide?

DR. GLICK: I meant that the lantern slide represented a magnification of seventy times, not as projected on the screen.

DR. MUDD: Was the magnification always obtained by photographic blowing up?

DR. GLICK: Yes.

MEMBER: Has Engström published any data regarding the mass? Of course there are data available about the dry weight.

DR. GLICK: There is a paper that just appeared in *Biochimica et Biophysica Acta* and I think there are some data on nuclei, but they are not isolated nuclei. You could not see them very well on the screen, but we were able to show nuclei in epithelial cells of the gastric mucosa. They had less mass than the cytoplasm, which is rather surprising. If you could see the original radiogram, you could see the dark nuclear areas, and from those areas one could actually calculate the mass. We did not calculate that mass here.

DR. GERR: Is there a question of homogeneity here again? If your particles might have a very high mass, you might get a sum total higher than that.

DR. GLICK: That does not enter into this type of measurement. The resolution of the film is the limiting factor here. This film is made by one company in Antwerp and has a resolving power of one micron, so that any structure larger than one micron will give a density area that can be quantitated without regard to other areas in the field. Now they are working on a new film that will resolve to one tenth of a micron. If

they can accomplish that feat, we may be able to see some of the cytoplasmic particulates.

DR. BUCK: I do not think I made it quite clear what I was getting at before, or whether I understood you originally. Your autograph is of the same size as the tissue. Do you use a parallel beam?

DR. GLICK: Yes; the tissue is placed in direct contact with the emulsion of the film, so that the recording is of the actual size, then subsequent photographic enlargement can be employed for convenience.

DR. BUCK: Would it be possible to use a point source, so that you can get a projected image?

DR. GLICK: It isn't necessary. What you want is a beam essentially parallel to fill the whole field of the section. You don't have to care about intensity, because if it is low you simply expose longer.

DR. BUCK: I was thinking about enlargement.

DR. GLICK: Technically it is more feasible to use subsequent photographic enlargement, which can be handled easier than trying to get divergent beams of X-rays.

DR. MUDD: I am still a little puzzled about this enlargement. Your calculation seems to indicate that you must use something like a thousand-fold photographic enlargement. Any plates that I am familiar with do not approach that. Would you mind giving us the name of your film, and would it be the same for electronmicroscopy?

DR. GLICK: This film is called Lipemann Film, manufactured by the Gevaert Company in Antwerp. I have written and asked if they could supply it to us in this country. They are working on it.

The enlargment given was that on the slide, not on the screen, and was of the order of 350 times. The grain size in this film is so small that you can enlarge three to four hundred times and still get fairly good detail.

DR. MUDD: That's marvelous.

Movements of Macrophages Studied With the Use of Thorotrast. J. W. WILSON and ELIZABETH H. LEDUC, *Brown University, Providence, R. I.*

Abstract. Colloidal thorium dioxide (Thorotrast) injected into the tail veins of mice is taken up by macrophages: particularly by those of the spleen, the Kupffer cells of the liver, and the macrophages in the walls of the sinusoids in the adrenal cortex. Since the material is radiopaque, its presence reveals the spleen and liver in roentgenograms. It is radioactive and can be quantitatively measured in aliquots of tissue by a Geiger counter. The radioactivity is not, however, great enough to produce extensive damage. Macrophages heavily laden with this material apparently continue to live for many weeks. Whatever damage they suffer must be slight at any one time, and constantly repaired.

Macrophages laden with granules of thorium dioxide can be followed in their movements. For example, in carbon tetrachloride poisoning the liver shows a characteristic central necrosis. Kupffer cells, previously loaded with thorium dioxide, move into the necrotic area around the central vein, leaving the intact peripheral area of the lobule clear of the material. In the adrenal gland, as the cells of the X-zone undergo the typical fatty degeneration, the macrophages move into it from the sinusoids of the cortex, leaving the cortex free of the material. In both the liver and the adrenal gland, subsequent injection of india ink shows that new macrophages have replaced the ones that have migrated.

Discussion

DR. G. B. WISLOCKI (*Boston, Mass.*): This paper is a logical sequence of a series of studies over the last forty years which have dealt with the experimental distribution of vital azo dyes and carbon particles in the body. The extensive and exact quantitative work on the distribution of manganese dioxide particles carried out by C. K. Drinker and his pupils is worth recalling in this connection.

There are some points in the present investigation that one would like to inquire about in the light of Drinker's experiments. Manganese dioxide in the first hours after injection is primarily captured by the lungs, but subsequently is gradually released to be redistributed in liver, spleen, bone marrow, and adrenal cortex, and traces in other areas, little eventually remaining in the lungs. Does your material behave similarly?

In these x-rays, the calcified bone interferes with the identification of your substance in the marrow. I reported years ago the deposition of carbon in red, but not in yellow, marrow, and I have noted particularly its presence in the endothelial cells of the active capillaries at the epiphyseal lines of growing bones.

DR. WILSON: Yes. It doesn't show in the X-rays. Actually it doesn't show in the X-rays of the adrenal gland, because there is not enough there. As far as the manganese dioxide is concerned, apparently the movement is a little different, because in the case of Thorotrast in less than 8 hours after the material is injected it is all in the liver and spleen. I know that it goes in very rapidly and in 2 hours you can get an X-ray picture showing the liver.

DR. WISLOCKI. Another interesting place to investigate by your method would be the placenta, with the questions in mind of placental transmission and the possible phagocytic properties of the trophoblast. I wish to ask whether you have made any observations on pregnant animals?

If I understood you correctly, you described the formation of thrombi around the central veins of the liver and at the junction of adrenal cortex and medulla. Those are not evident features, in my estimation, with india ink or manganese dioxide.

DR. ROBERT E. STOWELL (*Kansas City, Kansas*): Dr. Wilson showed pictures of a normal spleen that is quite homogeneous, from X-ray appearances. After the animal was treated with carbon-tetrachloride and azo dyes the X-ray showed a mottled appearance of the Thorotrast shadow in certain areas of the spleen. Morphologically what is the explanation of this? Is it an accumulation of macrophages in the spleen as in the other organs?

DR. WILSON. No, I don't think it is an accumulation, but a great deal of it is due to quite a drastic change in the spleen itself. This spleen that I showed the section of has been treated with an azo dye for about two hundred or more days, and the consolidation of the white pulp is quite striking, which produces the net-like form of the picture in the X-ray. Even in the normal animals the spleen is quite mottled because the Thorotrast is located in the red pulp.

Experiments on the Cytochemical Demonstration of Pentose Nucleic Acid. A. W. POLLISTER, MARTIN FLAX, MARION HIMES, and CECILIA LEUCHTENBERGER, *Columbia University, New York, N. Y.*

Abstract. Nucleic acids may be demonstrated by ultraviolet absorption if a blank, made by removal of the nucleic acid, is evaluated. In fixed cells the blank is from one-half to two-thirds of the total light loss at 254 mμ. Stains that color ultraviolet absorbing regions and fail to color the blank can be considered specific for nucleic acid. Azure A (0.1 percent, with alcoholic decolorization) appears to be such a specific method. It is reproducible, but quantitative use is complicated by deviations from Beer's law at higher concentrations. Pentose nucleic acid is usually distinguished from desoxypentose by a negative Feulgen reaction and susceptibility to ribonuclease digestion. The enzyme has been found to reduce the ultraviolet absorption of nucleoli and cytoplasm to the same extent as chemical extraction of all polynucleotides. Cytochemical use of ribonuclease probably cannot show more than that the digested nucleic acid is of the Feulgen-negative type, for the polynucleotide specificity of ribonuclease is questionable. Preliminary experiments indicate that ribonuclease removes desoxyribonucleic acid from nuclei of amphibian liver and cartilage, reducing the intensity of the Feulgen reaction. This Feulgen reduction is accompanied by an equivalent decrease in ultraviolet absorption; from which it appears that neither of these types of chromatin contains any considerable amount of pentose nucleic acid.

Ultraviolet Absorption Spectromicroscopy of Interstitial Cells of Testis Using Carbowax Embedding. HARLAN I. FIRMINGER, *National Institutes of Health, Bethesda, Md.*

Abstract. A preliminary study was made of the ultraviolet absorption characteristics of the lipoid material in interstitial cells in sections of mouse testes. Normal testes and stilbestrol-induced hyperplastic and neoplastic lesions were examined. The technique of embedding with the hydrophilic wax, Carbowax, was employed for obtaining thin sections of the tissues. Comparison of the results of ultraviolet absorption spectromicroscopy of the interstitial-cell lipoid in the tissues with that of natural sex hormones provided further support for the androgenic steroid nature of the interstitial-cell lipoid.

Discussion

DR. G. B. WISLOCKI (*Boston, Mass.*): This is an interesting contribution to the subject of the identification of this type of compound in tissues. In reference to Dr. Firminger's remark that hitherto there has been no specific histochemical proof for steroids, I would like to call attention to an article about a year ago by Ashbel and Seligman, who reported a specific histochemical reaction for carbonyl groups and published a picture of the testis of a dog in which the interstitial cells were stained by their procedures.

DR. A. M. SELIGMAN (*Boston, Mass.*): I might just say in comment on Dr. Firminger's paper that we have demonstrated that the lipoid of various endocrine glands contains ketonic material. This is not conclusive proof that the carbonyl-containing lipoid is indeed testosterone, but with the aid of other experimental evidence, such as the fact that testosterone was originally isolated from the testicles, one can conclude that the carbonyl-containing lipoid in the interstitial cells is testosterone. I think the kind of work reported by Dr. Firminger is very helpful in proving that what we are visualizing in the interstitial cells is testosterone.

DR. R. C. MELLORS (*New York, N. Y.*): Later on today we will show you another way of recording absorption spectra which will indicate the real complexity of this problem.

I think it will show clearly that there is not exact chemical specificity in this type of study *per se*, because practically all cells absorb ultraviolet light and absorb increasingly as the shorter wavelengths are studied.

One can distinguish between two classes of ultraviolet absorbing materials; one class is soluble in fat solvents, and the other class is not. Any number of tissues yield absorption spectra like this and contain compounds which can be removed by fat solvents. Finally the Carbowax itself is a good solvent for steroids.

DR. FIRMINGER: I bow to Dr. Mellors' greater experience in variety of tissues examined by the ultraviolet absorption technique. I would somewhat dispute his contention that the Carbowax is necessarily dissolving out the steroids in our material. I am sure that he has not had the experience with it, or with this type of pathological material, that we have.

I have not actually tested the solubility of steroids in Carbowax in the test tube. I would not doubt that they may be somewhat soluble. On the other hand, the solubility of material in tissues is entirely different from the solubility in the test tube. Certainly there is an immense amount of lipoid in the cells, and as far as I can tell morphologically, using several different sectioning techniques, I cannot detect any loss of lipoid by our Carbowax embedding.

I was interested in the solubility of this lipoidal material when I first started this study, and we tried putting sections in various fat solvents to see what the solubility of this material was. To my great amazement, using both fresh and fixed sections of material, we could leave them as long as overnight in chloroform, an excellent fat solvent, and when we did fat stains on them the next day, there were globules of fat in some of the cells. Granted that perhaps anywhere from 75 percent to 90 percent of the lipoid was lost, but there was still a fair amount of lipoid present.

I have no idea how specific this absorption spectrum is. I myself am skeptical, and until I have more data and can more thoroughly analyze it, I cannot advocate the

specificity of it. I feel that it probably won't be any more specific than say the phenylhydrazine reaction. When we started out we hoped that we would have more than one peak and actually get a good fingerprint, which I think was optimistic.

I would like to ask Dr. Mellors if he has any direct proof of the loss of steroids in tissue sections, and what that proof consists of?

DR. MELLORS: Carbowax is a class of polymers of polyethylene glycol. We have taken the problem up with the manufacturers of this material and have found that while it has no solvent effect on neutral lipids, it is a solvent for substances that contain the benzene ring. It is histologically useful in the sense that it will not dissolve neutral fat. However, if the adrenal gland is embedded with Carbowax there is a loss of the majority of material which has properties of the steroid class.

It is well known from many other points of view that complexes of lipids with cellular material or with extracellular material may be difficult to disassociate. The extraction of cholesterol in serum is an example. It is necessary there to use means of breaking bonds between the serum proteins and the cholesterol.

Now we have, over the period of several years, tried to approach the problem of steroids in cells from the point of view of their differential solubilities. It seems logical that one could arrange a series of solvents which vary from nonpolar solvents that are more like simple lipids, to polar solvents which have a higher ratio of oxygen to hydrogen. Thus far this work has not been successful.

Our work on the spectral studies of tissues has been limited by the fact that the thinness of the ordinary frozen section can be but 5 μ, and it requires careful technique to obtain them that thin. It is natural at first thought to turn to Carbowax embedding in an effort to prepare very thin sections, of a thickness of about one micron, because the scattering of light by customary tissue sections renders the interpretation of their absorption spectra very difficult.

If an adrenal gland, for example, is prepared as an unfixed frozen section and then dried in the frozen state it yields an absorption spectrum something like this. If that adrenal gland is prepared with the Carbowax method unfortunately some or much of the short wave length absorbing material is lost in sections of comparable thickness. We have obtained this result with several members and derivatives of the polyethylene glycols.

I think there is always the possibility that there can be some residual steroid that could be bound to cellular protein.

This problem is important to the group at large because it illustrates that we must constantly face the problem of analyzing mixtures.

DR. EDWARD D. DELAMATER (*Philadelphia, Pa.*): I would like to point out that by the very nature of the Carbowax polymers that probable solubility can be defined from many of these substances, if they will extract not only the higher polymers but other combinations that Dr. Manning and Dr. McCarthy have demonstrated to be extremely useful.

Then, too, in passing I would like to mention that in the last paper on the program a frozen section technique will be presented which is essentially simple and which I think will find application to your laboratory and to other laboratories.

A third point is that these same problems come to definition of new instruments, such as the phase microscope, so that in attempting to study bacterial and other cells in phase and the influence of such things as acid extraction of the cells, or at least acid hydrolysis of the cells, we are running into essentially the same problem.

With nucleic acid, perhaps because of the somewhat better-defined knowledge, which has been built up in the last 10 years, we are in a slightly better position to evaluate the specific influence of what we are doing. But I think that we still have the necessity of each of us attempting to define these new tools, using all the techniques we have available to us, including actual chemical analysis of the extracts.

DR. SELIGMAN: I think the techniques that have been discussed this morning by Dr. Firminger offer promise of settling a very important point that has been bothering those of us who have been using histochemical methods that visualize a specific func-

tional group, such as carbonyl. That is, that these carbonyl groups are not demonstrated in fresh tissue or fresh frozen tissue, but are demonstrated after formalin fixation. These findings have been very hard to explain. We have no notion of how the carbonyl groups of steroids are conjugated within tissues of the endocrine glands. Perhaps ultraviolet studies in fresh frozen tissue, using techniques such as here suggested or by the technique referred to by Dr. Coons yesterday, might indicate through a shift in absorption spectrum just how the steroids are conjugated in fresh tissue. Apparently the carbonyl groups of steroids are not free in fresh tissue, but are unmasked by formalin fixation.

DR. FIRMINGER: I would like to make one more remark. I did not mention the fact that we have carried out practically all the histochemical methods that have been recommended for steroids on our Carbowax sections, and I might say that they have all worked on this lipoid. Thus, if this lipoid is not steroid, then the lipoid we are calling steroid histochemically is not steroid.

DR. DELAMATER: Since in going up the polyethylene glycol series from low molecular weight to high molecular weight there is an increase in water solubility and decreased solubility in organic solvents, I would like to ask Dr. Firminger what molecular weight polyethylene glycol was used, and also what liquid medium was used to float the tissue sections on?

DR. FIRMINGER: We used a mixture, as has been described in the literature, of two parts of Carbowax "1500" and eight parts of Carbowax "4000." However, I must admit that we have varied that percentage according to the temperature. In the summer we found this mixture became so soft down in Maryland that we had to use a pure Carbowax "4000." It worked very nicely.

Also, we were unsuccessful in getting ribbons when we first started using Carbowax. So we played around for a while and found that we had to heat the carbowax on receiving it. After making up our mixtures we heated it to 175° C. for about 30 seconds, then put it in the paraffin oven, but we had to heat each new batch in order to get good ribbons.

DR. DELAMATER: Of course you changed the degree of polymerization by that heating process.

DR. FIRMINGER: I grant you that may be, but it works very nicely. As long as we get the results we are looking for, that is all we are concerned about.

DR. DELAMATER: Do you have to float your sections on water?

DR. FIRMINGER: We did float our sections on water to which we added a little Carbowax in order to prevent them from exploding, because of the lipoid content of the sections. You can use a wetting agent or some other means of reducing the surface tension, I am sure. That just happened to be the method we used.

DR. FIGGE (*Baltimore, Md.*): I would like to make this comment. In regard to getting sections in ribbons with Carbowax, it has been some 8 years since I played with this and ran into the same problem, and finally gave up the Carbowax method. But I have incorporated liquid latex and found that this greatly improved the ribboning. I offer this for what it may be worth.

DR. FIRMINGER: I think the main difficulty has been that the Carbowax is somewhat hygroscopic and it is necessary to remove some of the water. Dr. Mellors told me last night that they have succeeded in doing this by putting the Carbowax in the desiccator.

DR. STUART MUDD (*Philadelphia, Pa.*): I am just wondering whether some of these processes in which the thickness or thinness of sections becomes critical could not be aided materially by some of these methods of obtaining really thin sections which are in the process of elaboration by electron microscopy.

Aging Changes in the Collagenous Connective Tissue of Rat Skin. A Study With the Electron Microscope. JEROME GROSS, *Massachusetts Institute of Technology, Cambridge, Mass.*

Abstract. Examination of the connective tissue fibrils of the skin corium of rats ranging between 2 and 90 days in age with the electron microscope indicates that the argyrophilic "reticulin" of the newborn tissue has the typical morphology of adult collagen fibrils, namely an axial repeating period averaging 640 angstrom units and characteristic intra-period structure. These fibrils, although nonbranching themselves, have a strong tendency to group in long branching bundles. They differ from adult rat skin collagen in being roughly one half as wide. The gaussian width distribution curve for 200 fibrils from a 2-day-old skin ranged from 300 A to 700 A, with a peak at 500 A and a rare fibril more than 1000 A wide. A similar curve for adult rat skin collagen ranged from 800 A to 2000 A with a maximum at 1300 A. The mean values vary by about 200 A with different animals of the same age groups. The width distribution curve for fibrils from each of the skins of animals ranging in age from 18 to 60 days is spread irregularly from about 400 A to 2000 A, with the broader fibrils predominating in the older animal skins. Electron microscope examination of silver stained "reticulin" and collagen indicates the colloidal nature of the silver impregnation. The relationship of fibrils to ground substance and the nature of the latter will also be discussed.

Discussion

DR. G. B. WISLOCKI (*Boston, Mass.*): In reference to your last picture showing the tapering off of a fiber, there is a counterpart under the light microscope in that the fibers very evidently diminish in size. The reticulum fibers become invisible below a certain size and also fail to become silvered above a certain size when they have been compacted into collagenous bundles. Will you refresh my mind as to the resolving power of the light microscope?

DR. GROSS: The best resolution with the ordinary light microscope is about 0.1 or 0.2 μ. The resolution of the electron microscope, or at least the theoretical resolution, is somewhere from 5 to 10 angstrom units. But in actual practice we find that our resolution runs somewhere between 30 to 70 angstrom units.

MEMBER: Do you have any idea whether the silver is bound to specific groups in the fibril such as sulfhydryl?

DR. GROSS: There is no evidence from this work at all as to what chemical groups are where. Argyrophilia seems to be a colloidal affair anyhow; and whether it is an adsorption of some kind that is not related to specific groups, we do not know.

However, I might say at this moment that the field of electron staining is quite promising, although still embryonic. We have only two stains and these are nonspecific at the moment, but there are hopes that we can devise some specific stains for specific chemical groupings.

There is no evidence for sulfhydryl groups in collagen. Heringa and Weidinger reported 2 percent sulfur in newborn rat skin. However, they took the whole rat corium, which includes hair follicles and ground substance, which may contain much sulfur. They did not determine whether it is sulfhydryl or sulfate, and they just assumed that the 2 percent sulfur was obtained from collagen.

DR. JAMES HILLIER (*Princeton, N. J.*): I just want to compliment Dr. Gross on providing both interpretations on the thinning down of the collagen fiber. I have just been reviewing the biological uses of the electron microscope and it is astounding how often in the literature when there is a possibility of something going in or coming out, that the author invariably forgets the interpretation he is not interested in. Dr. Gross didn't do that.

DR. GROSS: I was a little bit afraid of showing that last picture, because it represents a rarity. I would just like to emphasize that in all the thousands of fibrils observed, I found about a dozen altogether. But it still may be significant.

Electron Microscope Demonstration of Bacterial Nuclei as Vesicular Structures.

STUART MUDD and A. G. SMITH, *University of Pennsylvania, Philadelphia, Pa.*

Abstract. A preparative procedure is described which permits parallel electron and light cytological observations to be made complementarily in studying bacterial nuclei. Osmic acid vapor is shown to be an excellent fixative in that it preserves the preexisting pattern of nuclear sites and enveloping cytoplasm, and accentuates contrast both in the electron and light microscopic pictures. Treatment with hydrochloric acid completely reverses, in the electron microscope image, the pattern of density contrast between nuclear sites and cytoplasm. The hydrochloric acid treatment removes the cytoplasmic basophilia, permitting clear visualizing in the light microscope of the stained nuclear chromatin.

The unfixed nuclei of young actively growing *Escherichia coli* cells have previously been shown to have less density than the enveloping cytoplasm (Hillier, Mudd, and Smith, 1949). The contrast in density between nuclear sites and cytoplasm is accentuated both in electron and in light pictures by fixation with OsO_4 vapors. These facts strongly suggest that the bacterial nuclei have a lower density (mass per volume) of solid matter to affect electrons and photons and to fix osmium than the surrounding cytoplasm. The nuclei also have characteristic shapes and do not merge with the cytoplasm. In the nuclear sites after appropriate processing Feulgen-positive bodies are found that stain with chromatin dyes and behave characteristically toward nucleic acid splitting enzymes. Expressed in the idiom of the cytologist, bacteria possess vesicular nuclei containing chromatin.

Discussion

DR. DAVID GLICK (*Minneapolis, Minn.*): In this connection it may be interesting to mention the result that Dr. Bayliss, Mr. Siem, Dr. Seligman and I got some years ago in trying to apply the Gomori enzyme stain to a variety of micro-organisms. Structures within quite a few organisms could be seen with the phosphatase stain. If these same organisms were placed in the electron microscope unstained and a picture taken, the pattern that was obtained looked exactly like that obtained with the phosphatase stain in the light microscope. It may be that the bodies you see with the electron microscope in these organisms, also possess phosphatase activity.

DR. MORIARTY: Regarding the caving in of the osmic acid, there is one peculiarity of fixation with osmic acid, and that is that you can watch it happening or you can do it by preparing specimens fixed by different methods and comparing them with the unstained and living organisms where you get contraction of the vesicles that we associate with these structures. You can actually see them.

DR. S. MUDD (*Philadelphia, Pa.*): You agree that there are vesicles then?

DR. MORIARTY: In thinking about them, we feel that there are. I think it is proved directly by that means.

DR. J. F. A. MCMANUS (*Birmingham, Ala.*): Dr. Winkler, in our Department of Bacteriology, has been applying the periodic acid method to bacteria, with or without acetylation, and materials can be demonstrated before acetylation that do not appear after acetylation.

I am not sure that he has tried *typhi*, but *Flexner* has that sort of safety-pin appearance which you showed with positive staining. *Enteritidis* has an appearance almost like a barber pole, and there are distinctive stainings, which I think have been mentioned by Pennington in the *Journal of Bacteriology*, with the periodic acid method.

If that is a nucleus, it has a sugar in it that reacts with the periodic Schiff method. This is not usual for the nucleic acids.

MEMBER: Dr. Mudd, have you tried replica to avoid complete desiccation of the cells?

DR. MUDD: Yes, Dr. Hillier developed the replica technique a number of years ago, and Wyckoff and others have done work with it. We do not feel that it tells us very much about internal structure; it shows wrinkles about surface contours and

that sort of thing, but does not show very much of the internal structure. Our particular problem is with the physical nature of the internal organ, the nucleus.

DR. C. F. ROBINOW (*London, Ontario*): I congratulate Dr. Mudd on his interesting electronmicrographs of hydrolyzed bacteria. The apparent increase in density of the nuclear structures which he showed had not been suspected and was puzzling. He pointed out that the chromatin structures of bacteria had been discovered and studied in many laboratories before they were recognized in electronmicrographs. Observations of the relatively low density of the sites of the chromatinic material in electronmicrographs of several species of bacteria had previously been published by Dr. Cosslett of the Cavendish Laboratory and me. The term "vesicle" applied to nuclei might evoke the picture of a rounded volume of more or less fluid material bounded by a recognizable membrane. We did not think that bacterial nuclei in general, including those shown by Dr. Mudd, conformed to this picture.

DR. MUDD: I should like to acknowledge the priority of Dr. Robinow as being the first one to show that these nuclear sites in the electronmicrographs were lighter, less dense, than the surrounding cytoplasm. Dr. Robinow first called my attention to this interpretation, which we have so strongly espoused. The difference between vesicles or no vesicles surely is a semantic one, and I have not felt that vesicles have to be rounded.

DR. DAVID L. DRABKIN (*Philadelphia, Pa*): I think that the term "vesicle" may have a different meaning to a chemist. A vesicle may be something that is not only round, but I somehow picture it as space or cavity. To what extent are these phenomena merely the expression of the effect of the size of the particle on scattering, because the technique appears to depend on scattering. The larger the particle, the greater will be the scattering. Originally you have particles of a certain size, but after you do various types of fixation and staining, the smaller particles in these so-called vesicles become aggregated. Can this be one explanation for the greater density?

DR. MUDD: I should much rather refer that question to an authority—Dr. Hillier.

DR. JAMES HILLIER (*Princeton, N. J.*): The point here is that while the three dimensional picture is missing, thickness as well as density is important. I consider that the light areas in the unstained and stained organisms might not represent a discrete structure but represent instead an area of lower density due to greater hydration. Shadowing shows that more material is lost from these areas on desiccation.

I could also answer an earlier question at this point by stating that there is good evidence now that it is the desiccation in open air that causes damage.

DR. MUDD: I think Dr. Drabkin's question was not completely answered in that he suggested that the state of aggregation of the absorbent material might be definitive in the electron scattering. As I understand it, and Dr. Hillier can correct me if I am wrong, the density of the electron image at any point is directly proportional to the thickness times the density of matter without regard to whether that matter is in fine or large aggregates.

DR. HILLIER: At any one point.

DR. ROBINOW: Regarding the polysaccharide material, as shown by the McManus-Hotchkiss technique, I have seen Pennington's slides. They show very clearly that the nuclei are in unstained regions between the sites of the polysaccharide material.

The Influence of Acid Hydrolysis Upon the Nuclear Structures of *Bacillus megatherium* as Demonstrated by Phase Contrast Microscopy and the Aldehyde Mordanted Basic Fuchsin Stain. E. D. DELAMATER, S. MUDD, and A. SMITH, *University of Pennsylvania, Philadelphia, Pa.*

Abstract. The effect of different periods of acid hydrolysis on the nuclear structures of *Bacillus megatherium* are demonstrated by means of phase contrast microscopy and by means of the aldehyde mordanted basic fuchsin stain elaborated by DeLamater and subsequently studied in bacteria by Smith. A change in the size and shape of

the nuclear structure, as well as the development of certain vacuolate structures within the bacillary cell, is noted as the hydrolysis period is prolonged. Corresponding changes are visualized by means of the phase contrast microscope.

Discussion

DR. C. F. ROBINOW (*London, Ontario*): It has long been known to workers in this field that *B. megatherium* must not be hydrolyzed at the standard temperature of 60° C. and that treatment with N/1 hydrochloric acid at 40° C. for 20 minutes gives pictures as clear and detailed as those which Dr. DeLamater has presented. (Dr. Robinow showed a lantern slide illustrating this.)

DR. DELAMATER: I might say that I am fully aware that my "neck is well out" and that I do this with some considerable trepidation, knowing the background of the subject, both from the point of view of optics as well as the limitation of optics, as well as those who have done so much in the basic work. But I feel that the tremendous amount of background that has been established is actually background material, and now, with the acceptance of the presence of a nucleus, whatever its form or configuration, the details must be worked out, and must be worked out with the development of newer techniques, and, if possible, with a comparison of all techniques; so that I do it actually with trepidation, which is no apology.

DR. LOTTI STEINITZ (*Columbia, Mo.*): Dr. DeLamater, I should like to ask whether it might not be better to regard the structures you have demonstrated in bacteria as chromosomes rather than as nuclei? I suggest the former for two reasons: genetic work on segregation and recombination in bacteria (Lederberg now at University of Wisconsin) has furnished evidence of only one linkage group so far, and the structures here shown are not like a vesicle in appearance but seem to be capable of assuming various forms and shapes that may or may not be related to a mitotic cycle. Whether we agree to call them nuclei or chromosomes for the time being is really immaterial as long as we keep in mind that the structural organization of hereditary material in bacteria might differ somewhat from that of higher plants and animals, although we would not expect the structures to divide by a method radically different from the known types of nuclear division.

DR. DELAMATER: Do you feel that Lederberg's work, at this stage of the development of our knowledge about it, necessarily eliminates the possibility of more than one linkage?

DR. STEINITZ: No, I definitely do not think so; but I think that one should use the simplest hypothesis.

DR. DELAMATER: Yes, but it is the first piece of work that has been done and that does not limit the possibility for expansion, so that perhaps the cytologic evidence, which not infrequently lags behind the genetic, may occasionally be ahead of it.

DR. S. MUDD (*Philadelphia, Pa.*): In reference to your question, Dr. Steinitz, Bowin's laboratory has also shown genetic recombination as well as Lederberg's, and both laboratories worked with *E. coli*. This is a much bigger organism. I don't see that there is any more reason to suppose that all bacteria have the same number of chromosomes than any other large category of organisms have the same number of chromosomes. Anyway there is very definite evidence, from the precision with which bacteria maintain their characteristics over thousands of culture generations and the fact that you do have mutations which do breed true, as in the beautiful work of Luria, Delbrück and all that group, which shows that you have to have some mechanism which allows for greater genetic precision than the idea of mere amitotic division of the chromosome body.

With regard to a single chromosome, Dr. Metz tells me that in the higher organisms there are some animals that have a vesicular nucleus or vesicular chromosome. It is a matter of semantics which you call it.

DR. STEINITZ: That is what I wanted to suggest, that maybe in bacteria, the chromosome is the nucleus. I did not mean to suggest that it could divide by mitosis.

Differential Centrifugation Combined With Microspectrophotometry in Localization of Cellular Bio-activity. D. L. Drabkin, *University of Pennsylvania, Philadelphia, Pa.*

Abstract. The differential centrifugation technique of A. Claude has been combined with our direct microspectrophotometric method for cytochrome *c* in a study of the localization of this biocatalytic chromoprotein in the parenchymal cells of "normal" and regenerating liver tissue from adult rats, maintained under standardized conditions.

The material subjected to differential sedimentation was liver parenchyma (mechanically separated from the fibrous portion) and suspended in 0.85 percent saline. The main fractions were: intact cells at 600×gravity, nuclear phase at 1500×gravity, mitochondrial phase at 9400×gravity, and remaining supernatant or microsomal phase. After removal of the unbroken cells, the total dry mass (corrected for NaCl) of cellular fragments and cytochrome *c* content of the suspension were each taken as 100 percent. The recovery of mass and cytochrome *c* in the nuclear, mitochondrial, and microsomal phases was of the order of 90 percent. No cytochrome *c* was found in the nuclear fraction, which formed 10 to 17 percent of the total dry mass. Sixty-eight to 73 percent, or most of the cytochrome *c*, was recovered in the mitochondrial fraction, 16 to 30 percent of the mass. Regenerating liver, in comparison with normal, had a lower content of fibrous tissue and a significantly larger mitochondrial fraction. It is probable that cytochrome *c* is exclusively localized in the mitochondria.

Discussion

Dr. J. F. A. McManus (*Birmingham, Ala.*): Dr. Drabkin's presentation is most interesting in view of the fact that in regeneration and in active growth the mitochondria do appear more numerous and smaller and might be expected to give a greater yield. It is also nice to see that optical or visual impressions can be measured.

Dr. A. B. Novikoff (*Burlington, Vt.*): I think that this paper, which I would prefer to call essentially biochemical rather than histochemical, can be used to indicate the need for care in accepting biochemical data as criteria by which we measure the validity of histochemical methods.

Dr. Drabkin has used physiological saline in which to disrupt the cells and sediment the particulates. This medium had been employed some time ago by Schneider and co-workers but they discarded its use largely because of the high degree of agglutination of the mitochondria in it. More recently, Schneider and Hogeboom have reported data on cytochrome *c* with homogenates in isotonic and hypertonic sucrose. Even in these media, where mitochondria do not agglutinate, they found some, albeit very low, cytochrome *c* in the nuclear fraction.

I therefore find it difficult to see how in Dr. Drabkin's experiments, where the nuclear fraction must undoubtedly contain mitochondria, this fraction has no cytochrome *c* whatsoever, while the mitochondria have some 70 percent of the total content. Has Dr. Drabkin examined the nuclear fraction microscopically, especially with the phase microscope? Has this fraction been analyzed for pentosenucleic acid?

I raise these questions to indicate to an audience composed largely of histochemists that among biochemists engaged in this particular type of work considerable disagreement might exist. Although undoubtedly biochemical data can often be used as a yardstick for judging histochemical results, these data, too, must be scrutinized carefully. There are pitfalls in biochemistry as well as in histochemistry.

Dr. H. Shapiro (*Philadelphia, Pa.*): I find these observations of interest because they are in line with some respiration measurements that we made on single living cells (eggs of the sea urchin, *Arbacia punctulata*) which were centrifuged so that the intracellular granules became packed in easily differentiable layers, and if you centrifuge long enough and with adequate centrifugal force, you can separate the cell into two parts. The two fractions are viable, because they can be fertilized and will undergo development. The centripetal portion is that which, from the standpoint of the histochemist, contains the mitochondria and apparently therefore the cytochrome *c*

as well. It is that portion which, when fertilized, shows an increase in oxygen uptake of about 140 percent, which is similar to the activity of the whole cell, on fertilization (*Jour. Cell. and Comp. Physiol.*, 6: 101, 1935).

When you take the centrifugal portion you find that the rate of oxygen uptake on fertilization shows relatively little change (a 20 percent increase).

In other words, it would appear from these observations that the increase in oxygen uptake due to fertilization is associated with the cytochrome *c*, that is, it is the light cell fraction which contains the mitochondria, and which does show the large increase in oxygen uptake. These are observations that were not taken on the sort of brei that you worked with, but on intact cells which remained living for a long period of time, with all the morphological and physiological conditions remaining normal. Other considerations may be introduced to account for the activity of the centrifugal fragment, but these are discussed elsewhere.

DR. DRABKIN: I am not certain, but I think Dr. Novikoff may have created an impression that our conclusions differ from those of Schneider and Hogeboom. They do not. Their conclusions also are that the cytochrome *c* is limited to the mitochondria.

There is no pretension at this stage that any clear-cut separation has been accomplished. I have no doubt whatever, for example, that what I call the nuclear phase still contains some unbroken cells and may possibly contain a negligible amount of the larger particle aggregates. But with this technique you do make a sufficiently clear separation, and when you make a smear you can actually identify the materials. In the case of the mitochondrial phase, one finds no nuclei whatever.

With reference to the question of zero concentration of cytochrome *c*, nothing is ever zero. It is zero within the limitations of our ability to measure it by our technique which happens to be the best at present available for this type of measurement.

In the actual method of measurement the mitochondrial fraction is "processed" in order to analyze it for cytochrome *c*. The question that comes up is why shouldn't one determine directly the cytochrome *c* in the particles, and obviously, eventually that should be done. Accurate measurement of cytochrome *c* in a turbid suspension of cell particles is at present not possible, owing to the complicated nature of the nonspecific absorption due to scattering, *etc.* You must choose between the alternatives of accuracy of measurement, or having the material close to its biological state.

Eventually, we hope to be able to make more clear-cut separations and also to study the spectra of these materials within the particles, but I think the fact that we mash up the tissue and separate the constituents does not necessarily differentiate this type of study from a histochemical technique. We still are studying cellular structures and we are, I think, obtaining a closer approach than has heretofore been possible. The fact that a very large group of important cellular enzymes is localized in a particular portion of the cell has been more or less proved. The amount of cytochrome *c* in the nucleus is negligible, whereas the concentration of this pigment in the mitochondria is high.

Studies of Cells With the Reflecting Microscope. ROBERT C. MELLORS, *Sloan-Kettering Institute, New York, N. Y.*

Abstract. Recent advances in microscopy, among which is the development of achromatic reflecting optics, make it possible to study many fundamental biochemical processes in the cell in a manner not heretofore possible.

The purpose of this communication is: 1) to describe the use of a spectroscopic method which permits the recording of the visible and the ultraviolet absorption characteristics of cells within a period of a few seconds; 2) to illustrate the preliminary application of this method to the study of exfoliated cells, bone marrow smears, and tissue sections; 3) to indicate to what extent ultraviolet spectroscopic studies can be made of living cells without injuring them; and 4) to present some quantitative microabsorption data for living cells in the resting and the dividing states.

Discussion

DR. A. W. POLLISTER (*New York, N. Y.*): I suppose that if he had spoken a little longer, since he mentioned Commoner, Dr. Mellors would have discussed the possible influence of molecular orientation on those different values of the resting and dividing nuclei—since I heard Dr. Mellors present that point of view in a paper last Christmas.

It may be of interest to this group to recall that Commoner, in his recent theoretical discussion of the possible influence of orientation of nucleic acid molecules on absorption measurements of cells, did not present any evidence of his own that, actually, there is in cells any such approximately crystalline orientation of nucleic acid. His only supporting evidence consisted of an interpretation of the data on ultraviolet absorption, which had been presented by the Caspersson school. This interpretation is open to some question. For his average values Commoner lumped together absorptions of a great variety of objects, such as nuclei, nucleoli, and cytoplasm. For example, from Thorell's data Commoner lumped together blood cells of all sorts: mature and immature white and red cells, as well as cells from pernicious anemia. If Thorell's data are examined, it will be noted that there are many whole tables, in which there are no values in the range of 0.300, to which Commoner attaches most importance. Furthermore, in most of these ultraviolet absorption data which Commoner selected he did not take into account the fact that a large proportion of the light loss which the Caspersson school found was not due to specific absorption, but to light scattering and internal reflections—which have nothing to do with the actual specific absorption of the nucleic acid. The whole case for influence of molecular orientation on absorption in cells falls to the ground if you do subtract that sort of light loss; and you are perfectly justified in doing this in many cases because the authors do present the data on nonspecific light loss, along with the absorption.

We have gone over all our data in the visible and ultraviolet spectra, looking for a possible effect of molecular orientation on absorption. You can readily detect an effect if you study the highly oriented cytoplasm of striated muscle; there is no doubt about that; and the only time we started a study of muscle, we abandoned it soon because of such difficulties as this. But except for that, there is no indication of orientation influencing the absorption of these color reactions, or of natural ultraviolet absorption. That is not so remarkable, because there must be a very high degree of orientation, as in muscle, to cause much of an effect on absorption; and it is of course unlikely that a resting nucleus or a metaphase chromosome has such a high degree of orientation of nucleic acid molecules. It seems absurd to anyone who knows how a nucleus or chromosome looks.

Furthermore, if you stretch nuclei, even as violently as you do in a Waring blendor when you prepare what are called isolated chromosomes, even these stretched out nuclei show no marked birefringence indicative of molecular orientation.

The upshot of the matter is that, if anyone is interested in knowing whether orientation of molecules of nucleic acid or other substances actually has a detectable effect upon cellular absorption measurements, there is as yet no demonstration of it except in muscle. In a more recent comment, Commoner points out that even in the extreme case of a mosaic virus nucleoprotein crystal the Millon reaction for protein shows no evidence of an influence of orientation on absorption.

If one is to interpret the data which Dr. Mellors has just presented as an expression of the influence of orientation, one would assume that the increase in absorption at metaphase must be due to a disorientation of the nucleic acid that progresses during the prophase of mitosis—since the cells in division absorbed a great deal more, *i. e.* showed a much higher extinction, than did the resting cells. As an alternative explanation, I should like to ask Dr. Mellors to what extent he thinks the fact that a cell in tissue culture, when it is going to divide, draws in its processes, rounds up, and thus becomes a great deal thicker, has to do with this matter of absorbing a great deal more at metaphase. Everyone who has seen motion pictures of dividing cultured cells must have been struck by the fact that this is exactly what marks the onset of cell division—that cells round up, draw in their processes, and therefore become thicker,

since there is no evidence of change in mass of the cell. This greater thickness must of course greatly increase the absorption at metaphase.

DR. MELLORS: We are now studying the problem of space-orientation of cellular material in living cells with polarized ultraviolet light. As I see it much of what has been said is based on the analysis of fixed cells. I think that one can interpret studies of fixed cells with polarized ultraviolet light at 260 millimicrons as indicating no orientation of ultraviolet chromophores at that wavelength except in striated muscle.

The increased absorption at 260 mμ which occurs during mitosis is dependent upon at least four factors: (1) when the extended fibrils begin to spiralize, there is a shortening of the fibrils and a packing together of the ultraviolet chromophores, (2) the randomness of the distribution of chromophores may be increased, (3) at the time of reduplication of the chromosome there is a doubling of the number of potentially absorbing groups and (4) a change occurs in the shape of the cell as suggested by Dr. Pollister.

DR. R. E. STOWELL (*Kansas City, Kans.*): I wonder if Dr. Mellors could clarify the size of the area he is measuring. I understand, in terms of length, that this is adjustable. What about the width of the area being measured? Has the problem of scattering and dispersion been taken into consideration and, if so, in what way?

DR. MELLORS: The dimensions of the area that we study is variable in either the vertical or the horizontal dimension. In the spectral recordings that utilize a mercury arc as source, one wants to see as much of the cell as possible so that the slit of the spectrograph is opened up to admit a lot of light. The dispersion of the spectrograph decreases as the entrance slit is opened up so that the images of the bright lines will eventually overlap, and this becomes a limiting factor. Now there is latitude in this regard with some of the bright lines of the mercury arc, but with the hydrogen arc there is a different situation. Since the source is a continuum, one desires to narrow the slit width in order to obtain sufficient dispersion. This in turn decreases the amount of energy admitted through the slit. The exposure times for photographic recording then become prolonged and limiting.

To summarize, one can analyze areas which are dimensionally equivalent to the limits of resolution of this objective. An area of about 0.1 square micron can be studied by this technique using an ultraviolet sensitive plate such as Eastman's 1030UV.

I might say that from the point of view of densitometry one can also study that area, in the enlarged image, of course.

DR. D. L. DRABKIN (*Philadelphia, Pa.*): I would like to say a few words. I am sorry that I missed a portion of Dr. Mellors' paper. I thought the business meeting was going to come first, and not being a member of the Society I stayed out; but I heard him talk this morning.

I agree with everything he said this morning, but I don't know whether he had an opportunity to discuss further errors in the application of optical techniques to tissues or tissue slices. However, there is an interesting point that should be mentioned. I believe many of you may have seen the criticism which appeared in *Science* of the interpretation of findings by the spectro-cytological technique. Paradoxical situations may arise if you work with a pigment such as cytochrome *c* and work in the visible region, you deal with a substance which has such a striking and unique spectrum that, even in the presence of large impurities, the spectrum stands out like a sore thumb, and accurate measurement and interpretation become possible.

On the other hand, I suppose you get into another problem in which you are interested not only in cytochrome *c* but in the total iron. You promptly discover that your "isolated" extract of spectroscopically pure cytochrome *c* contains five times as much iron as cytochrome *c*. By this means you disclose that you have present other iron compounds that do not interfere spectroscopically with cytochrome *c*, when analyses are performed in the visible spectral region.

In work in the ultraviolet spectral region, the situation is very different. Here, almost countless different substances have specific selective absorption. Superficially, it may appear that the ultraviolet region should be a favored region for spectroscopic

differentiation. Actually, you have an embarrassment of riches. The paradoxical result is, that owing to the overlapping selectivity of numerous compounds, as I think Dr. Mellors has intimated, it becomes sometimes virtually impossible to separate very closely similar substances, particularly in the presence, when dealing with tissues, of an extreme amount of scattering.

DR. MELLORS: My apology to Dr. Stowell for not answering his question. Light will be scattered. The absorption which occurs at 240 millimicrons and on out becomes a great complex of the end absorption of organic compounds, the increasing factor of light scattering, and so forth.

DR. POLLISTER: This problem, which of course is just exactly as outlined, is a good reason why it is extremely difficult to work with living cells, using the method of photometric chemical analysis.

The point I have been trying to make in a number of publications for the last 2 or 3 years, is that from the standpoint of nucleoprotein analysis it is much better to work with fixation, and chemical extractions and enzymatic digestions, of the sort I discussed this morning. If, for example, you fix in acetic alcohol you immediately remove all sorts of things that will complicate the absorption, and there is abundant evidence that nothing but nucleic acids and proteins are left in the tissue. In most fixations like that, you then deal with the absorption of that nucleoprotein mass; measure the absorption, extract something, and you see what absorption is left. It seems that is the only possible approach—an approach that cannot be used on living cells at all. I must say that such a thing is practically limited, of course, to nucleic acid and proteins.

I would like to say one more thing about what Dr. Mellors had to say about fixing material. He said that it was well known that in fixed material there was no orientation. He did not say, except by implication, that in living cells it would prove that there was extensive orientation, although I thought he meant to imply that. But as a matter of fact, when you deal with nucleoproteins, the evidence is that fixation, if anything, will increase the orientation. That was pointed out long ago by Caspersson—that there was evidence of increased orientation in fixed material, and it is a matter I have observed myself very readily. If you take nucleohistone fibers, very good precipitated nucleohistone fibers, and observe their birefringence, it is low; but if you fix those same fibers and dehydrate them, then the birefringence is much higher. So, if anything, the fixation process should increase the birefringence.

DR. MELLORS: Most of what Dr. Pollister has been talking about is related to the birefringence of form which is seen in the polarizing microscope with visible light. Our interests are currently directed toward the study of orientation with polarized *ultraviolet* light.

Optically the best cell to analyze is the healthy living cell, in tissue culture thinly spread out because of stereotropism. It is the one cell upon which reliable microabsorption measurements can be made because every evidence points to the fact that the light scatter that occurs at phase boundaries and that is enhanced by any precipitating or fixing method is minimal in the healthy, resting cell.

DR. S. MUDD (*Philadelphia, Pa.*): Just one word. I think all those who have worked with the electron microscope have realized, as we never realized before, what drastic changes the ordinary histological and cytological fixatives do bring about in the ordinary protoplasm. I won't stop to elaborate on that, but surely most fixatives other than OsO_4 change the picture from any reasonable facsimile of the living state.

A Modified Freezing-drying Apparatus. R. E. Stowell, *University of Kansas, Kansas City, Kansas.*

Abstract. The preparation of tissue by freezing-drying methods is preferred for many histochemical studies because of the avoidance of deleterious action of chemical fixation and extraction of aqueous and lipoid solvents in fixing and dehydrating fluids.

Freezing-drying apparatus has been designed that permits preparation of tissues from a few hours to a few days depending upon the size of the specimen. Liquid nitro-

gen serves as a most efficient moisture trap and, in conjunction with a small heating element beneath the tissue, cools the tissue to any desired temperature down to −80° centigrade. The mean free path from the specimen to the moisture trap is shorter than with most other similar apparatuses. A mechanical forepump and oil-diffusion pump provide pressures down to 10^{-5} mm. mercury, and the equipment gives good pumping efficiency. The temperature of the specimen during drying is read with a thermocouple. Paraffin infiltration is started without disassembling the equipment and is completed in a few minutes. Most histochemical reactions can be applied directly to sections of frozen-dried tissue cut from the same block.

Discussion

DR. DAVID GLICK: I think that Dr. Stowell's equipment represents some improvement over most of the existing equipment now in use. There are a few questions that I should like to ask. One is that I did not notice any provision for measuring the vacuum, and if you do not have some sort of vacuum gauge how can you tell when the dehydration is complete?

Then I would like to know what kind of seal you have between that glass tube and the stainless steel over it, and what is the shortest time required for dehydration, according to whatever criteria you employed, to get satisfactory results?

DR. STOWELL: For the vacuum measurements, instead of using one of the rather expensive ionization gauges we have been using an inexpensive Tesla coil with which one can tell if one has a vacuum below 10^{-3} mm. Our equipment is designed to pump down to 10^{-6} mm., but we probably don't get that high a vacuum. As long as we are down to 10^{-4} mm. drying conditions are quite satisfactory.

The type of seal between the glass tube and the metal tube is important. In our earlier equipment, we had a semi-ball and socket joint and that was difficult to separate and was also a source of leaks. But recently various types of silicone rubber have been developed that are especially good for vacuum work. In this instrument the seal consists of a simple ring of silicone rubber that requires no lubricant and that one can easily pull apart as soon as the leak valve on the system is opened to allow the air to enter. The seal is efficient, and easy to break, and we have had no trouble with leaks from this seal.

The drying time, of course, varies with the size of the block of tissue used. With this technique it is important to use thin pieces of tissue to avoid large amounts of ice-crystal formation. We have found that 5 hours is quite satisfactory for drying tissues. This time is measured from the time the tissue is put in the instrument until it is taken out in the infiltrated condition, including the time it takes to pump down the vacuum.

DR. HERBERT MESCON (*Philadelphia, Pa.*): In an attempt to obtain a simple apparatus to answer the same purpose that Dr. Stowell has set forth, we came across Adamstone and Taylor's technique, which used the old type of microtome found around most medical schools and hospitals, to which we attached the stage of an ordinary freezing microtome and two pieces of sheet copper. Using dry ice to keep the blade of the microtome cold, sections could be cut, and would remain frozen on the blade, and we then transferred them to a slide still kept frozen until they came in contact with the slide. Then the slide was immediately immersed in whatever fixative, stain, or substrate that was desired. In that way, within 2 to 5 minutes after a piece of tissue was obtained, either biopsy tissue or an animal was killed, we were able to obtain a frozen section from 2μ to 5μ thick. We could cut liver at 2μ. That has been the experience also of Dr. Adamstone and of Dr. Taft. We have been able to cut skin at 5μ to 7μ.

The sections are comparable to paraffin sections in cellular detail. They do not have any of the wrinkling common in ordinary frozen sections.

The entire cost of the apparatus is the dry ice necessary, which runs about five cents a pound. An entire day's operation is about twenty-five cents.

The apparatus itself, if you can find an old microtome around, is just the machine with two pieces of copper, and we find that works quite well, perhaps not for all things as well as Dr. Stowell's technique. But we certainly get comparable distribution of enzymes, fats, and other material in tissue that compares favorably with the pictures as shown by Dr. Stowell's freezing-drying technique.

DR. STOWELL: Dr. Taft and others working in our laboratory have used this technique of Adamstone and Taylor and found it useful. As presently employed I doubt that either technique will do for all purposes. We still have to use a number of techniques for preparing tissues for different types of observation.

DR. WILLIAM F. WINDLE (*Philadelphia, Pa.*): Recently we have constructed an apparatus in the Department of Anatomy in the University of Pennsylvania, and I do not know whether Dr. Stowell had an opportunity to see it. Dr. Liu, standing in the back of the room, has developed it at a cost I believe of about $300, and I think it has some features in common with yours, though it is different in other ways. If there is anyone else here interested in developing low-cost freezing-drying equipment, I believe Dr. Liu could arrange to show them the machine he has recently constructed.

MEMBER: What is the highest temperature you maintain in your freezing-drying equipment?

DR. STOWELL: We usually maintain the temperature at −30°C until we feel confident that the tissue is virtually completely dry. Our instrument is adjustable so that we can operate it at a lower temperature, but that prolongs the drying time and doesn't seem to be indicated for the things in which we are particularly interested.

MEMBER: We have built equipment in which we use dry ice to cool the tissue.

DR. STOWELL: A number of other people have done similar things. Gordon Scott, used dry ice and acetone. Taylor published a modified freezing-drying apparatus based on the use of acetone and dry ice. We have not tried to do that. I think that one of the principal advantages of this system is the very efficient moisture trap that is located close to the tissue, so that the molecules usually travel only a few centimeters before they are trapped. Another feature is the rapid and efficient pumping system.

DR. GLICK: I might add that from our own experience we find that the pieces have to be cut as small as two millimeters on a side. When they get larger, the center of the piece does not show the same morphology as the other part.

DR. STOWELL: We found that by laying the tissue on aluminum foil before freezing in isopentane one can use a thin sheet of tissue and it will not become distorted as much as usual. The aluminum foil is, of course, a very good conductor of cold. Using aluminum foil we have been able to use pieces of tissue up to a centimeter in cross section but we try to keep our pieces less than a millimeter in thickness.

DR. CHAN-NAO LIU (*Philadelphia, Pa.*): In our equipment we use a glass joint in the vacuum system and find it works satisfactorily.

DR. STOWELL: We use a gasket of special silicone rubber and have found it much better than a glass joint. The ground glass joint in our original equipment was occasionally a source of leaks in our vacuum and stuck so that the glass equipment was broken several times in removing the tissue.

DR. GERTRUDE D. MAENGWYN-DAVIES (*Baltimore, Md.*): May I ask how long you expose the tissue to 56°?

DR. STOWELL: A few minutes. We take the apparatus apart as soon as the paraffin is melted because we don't want to have our paraffin too hot in the vacuum, transfer it for a few minutes to a second paraffin change in the oven, and then embed it.

DR. MAENGWYN-DAVIES: You do expose it to 56° for something like 10 minutes?

DR. STOWELL: Yes.

DR. GLICK: You break the paraffin—rather, the vacuum, while it is still liquid?

DR. STOWELL: Yes, we remove the tissue and the paraffin from the instrument while it is still liquid.

The Histochemical Differentiation Between Types of Cholinesterase. G. B. KOELLE, J. S. FRIEDENWALD, ETHEL S. KOELLE, and WARREN WURZBACHER, *Johns Hopkins Hospital, Baltimore, Md.*

Abstract. Cholinesterase (ChE) activity can be localized histochemically by incubating frozen sections in a medium containing acetylthiocholine (AThCh), copper sulfate, and glycine, and subsequently treating with ammonium sulfide solution (Koelle, G. B., and Friedenwald, J. S., *Proc. Soc. Exper. Biol & Med.* 70:617, 1949). Following a study of the rates of hydrolysis of several substrates by pure and mixed enzymes, and of the degrees of inhibition produced by different concentrations of di-isopropylfluorophosphate (DFP), the method has been modified to permit distinction between sites of specific and nonspecific ChE activity. The former is localized in cat tissues by first treating sections with 10^{-6} M. DFP for 30 minutes, then incubating in a medium containing AThCh plus the same concentration of inhibitor. Under such conditions, the nonspecific fraction is almost completely inhibited, whereas specific ChE retains approximately sixty percent of its normal activity. Butyrylthiocholine is used as the substrate for demonstrating nonspecific ChE activity. Findings will be described and illustrated in the following tissues: motor cortex, spinal cord, striated muscle, sensory ganglia, autonomic ganglia, adrenal gland, salivary gland, and intestine. Their implications in respect to the physiological roles of the two types of enzymes will be discussed.

Discussion

MEMBER: Have these methods been used to study the endocrines?

DR. GEORGE B. KOELLE: No, they have not yet; and the greatest difficulty is something that Dr. Friedenwald is going to mention later, that there is great difficulty in trying to apply these methods quantitatively. We are hoping to do studies like that in the future, but I think that they are going to be extremely difficult inasmuch as the amount of cholinesterase present in the endocrines in that condition is two or three times as active, or let us say a third as active as normal. Unless the degree of difference were that great, I think it would be extremely hard to specifically say that was the case.

Investigation of the Lead Nitrate Method for Histological Demonstration of Acid Phosphatase. J. B. GOETSCH, *Yale University, New Haven, Conn.*

Abstract. The technique for histological demonstration of acid phosphatase notoriously produces inconstant and hence unsatisfactory results. Investigation of the details of the technique uncovered several critical steps which when controlled gave consistent results. Such matters as preparation of the substrate, the use of a low melting point wax with excellent cutting properties, mounting of the tissue sections, storage of block and paraffin ribbons, and, finally, details of incubation were found to be of importance. Chilled acetone fixation and frozen-dried preparations were compared as to the preservation of enzyme in control studies. An approach to a quantitative estimation of the relative activity or amounts of the enzyme over its distribution in the tissue is discussed.

Discussion

DR. ANITA ZORZOLI (*St. Louis, Mo.*): Do you recommend room temperature fixation or icebox fixation, and secondly, how do you clean the slides upon which you put no fixative?

DR. GOETSCH: I do not believe that it makes any difference whether the fixation is done at room temperature or not. Since it was originally recommended that we do it at icebox or refrigerator temperatures, we do that routinely; but actual experiments show there is no demonstrable difference. After the exposure to acetone, room temperatures can be used with impunity.

Regarding the cleaning of slides, they are cleaned either with a chemical cleaning agent or just the routine method of washing them and soaking them in acid alcohol, washing them again in distilled water, and drying them. There is no special routine that we use.

DR. H. MESCON (*Philadelphia, Pa.*): I would like to say that our findings on skin confirm in part Dr. Goetsch's presentation, but even more strikingly so. Two years ago Dr. Glick reported on 50 consecutive biopsies of skin and found that no acid phosphatase was present, using the Gomori technique.

Using our frozen section technique that I just outlined, we were able to demonstrate grossly black tissue, if we put it directly into substrate within 3 minutes after biopsy was obtained. If we fixed it in alcohol for about 3 minutes and then went into the substrate—in other words a section was cut right on the slide into acetone before it thawed or as it thawed—we got a better histologic picture but less phosphatase. And if we ran the section on the slide through cold acetone, then alcohol, xylene, paraffin, xylene, substrate, we then got no acid phosphatase, which was as you have done.

DR. GOETSCH: I should like to point out that it seems to us fairly definite that the crux of the matter is in the adequate fixation and dehydration of the tissue. There is at least chemically a great deal of inactivation that takes place up to that point, but once that has been accomplished, then the residual activity seems to be quite stable and as long as these simple bounds are not transgressed, uniform preparations are possible.

DR. DAVID GLICK (*Minneapolis, Minn.*): I would like to make one small correction of the comment that was just made. We did not say that the acid phosphatase was not in the skin. We said that using the methods we employed we did not demonstrate it.

DR. ROBERT MOWRY (*Boston, Mass.*): I wonder how important you found pH control, and what pH control you used; and secondly, did you have any interference by alkaline phosphatase operating in that pH?

DR. GOETSCH: We routinely used a pH of 4.3 to 4.4. We have not attempted to run any experiments on the finding of alkaline phosphatase at that pH. That did not seem to be part of our problem at the moment. From the alkaline phosphatase we have found no interference at all.

DR. MOWRY: Did you use buffers or electrometric control at 4.4?

DR. GOETSCH: That was a buffer substrate that we checked electrometrically. We used a regular substrate.

MEMBER: I wondered whether any difference has been noted in the use of various substrates?

DR. GOETSCH: We have not used different glycerophosphates. The only thing we did on that was to check the various commercial preparations. We used five different preparations from three different companies. We varied the concentration quite a good deal on a very dilute substrate and found no significant change in the histologic picture.

Some Histochemical Observations Concerning the Existence of More Than a Single Type of "Alkaline" Phosphatase. V. M. EMMEL, *University of Rochester, Rochester, N. Y.*

Abstract: Considerable biochemical evidence supports the view that the "alkaline" phosphatase of intestine differs from that of the kidney. Various histochemical studies have affirmed, and others have cast doubt on this conclusion. In studying by means of the Gomori technique the resistance of intestinal and renal alkaline phosphatase to denaturation by hydrochloric acid, it has been found that the former is destroyed by treatment for ½ hour at about pH 2.0; whereas the latter is destroyed at about pH 2.6. The addition of M/100 magnesium sulfate to the hydrochloric acid reduces the concentration of the latter, which is required to inactivate both renal and intestinal phosphatase, but does not alter their relative susceptibility. The "alkaline" phos-

phatase of the intestine is in both instances more resistant to acid denaturation than is that of the kidney.

It has previously been shown that the "alkaline" phosphatase of the intestine is more sensitive to cyanide inhibition than is renal phosphatase—just the reverse of their relationship in respect to susceptibility to acid denaturation.

These histochemical observations cannot at present be interpreted in terms of a single enzyme differing only in concentration in these two organs. They offer histochemical support for the view that renal and intestinal "alkaline" phosphatase are not identical.

Discussion

DR. ARNOLD M. SELIGMAN (*Boston, Mass.*): May I make just one comment? I would like to say that there may be some evidence from our work of still a third variety of alkaline phosphatase: alkaline phosphatase that is destroyed by acetone fixation is preserved when tissue is fixed in cold formalin for 24 hours. This is brought out particularly well in brain tissue. If acetone-fixed tissue is used, the only alkaline phosphatase that can be demonstrated is in the walls of certain blood vessels. After fixation in formalin the blood vessels have very little alkaline phosphatase and the brain cells are very richly stained for alkaline phosphatase. This constitutes evidence of a third variety of alkaline phosphatase, one which is ordinarily destroyed by acetone fixation.

Another interesting point is that this formalin-resistant phosphatase is not activated by magnesium ions, whereas the acetone-resistant phosphatase is activated by magnesium ions.

A Histochemical and Biochemical Study of the Alkaline Phosphatase Activity of Bone of Scorbutic Guinea Pigs. ANITA ZORZOLI and E. M. NADEL,[1] *Washington University, St. Louis, Mo.*

Abstract. Young adult guinea pigs were maintained on a scorbutogenic diet for 26 days and then sacrificed for study. The proximal end of one tibia was fixed, decalcified, embedded in paraffin, and sectioned for study of the histochemical localization of alkaline phosphatase. The other tibia was weighed, homogenized in distilled water, and allowed to autolyze at room temperature for two days. A suitable aliquot was used for the chemical estimation of phosphatase activity.

Both chemical and histochemical methods demonstrated a significant decrease in phosphatase activity of the scorbutic animals as compared with the normal controls. Expressed as micrograms of phosphorus liberated per gram of tissue per hour of incubation, the experimental animals had an average value of 13.98 and the normal controls of 30.91. The decrease in enzyme activity was widespread throughout the bone. This was especially true of the region of articular cartilage bordering on the secondary zone of ossification and in the columnar and hypertrophic cartilage regions, where the intracellular and extracellular phosphatase activity is normally very high. As indicated by the degree of blackening, the altered osteoblasts of the scorbutic animals showed little or no phosphatase activity.

Additional serial sections were stained with hematoxylin and eosin thionin, and by the periodic acid Schiff method, with and without prior treatment with hyaluronidase.

Discussion

DR. DAVID GLICK (*Minneapolis, Minn.*): In opening the discussion of the last two papers I should like to comment about the interesting differences in the properties of the enzyme from the kidney and from the intestines. I think it is a little dangerous to conclude that you have different enzymes based on this kind of evidence, not that I doubt that these differences are real; but I once spent about a year studying the

[1] Surgeon, U. S. Public Health Service, National Institutes of Health, National Cancer Institute.

changes of pH-activity curves of enzymes and it is amazing how many extraneous things that may be present in the solution can alter the shape of that curve.

The difference in the curves from the two sources was distinct, but it is quite possible that the variation in the materials present in these extracts might well account for the difference in the curves.

If there is a real difference, I think that it would have to be established by a more rigorous chemical method.

I should like to ask Dr. Zorzoli if this vitamin C is an activator, and whether the lower activity in the scorbutic material might not be explained just by the deficiency of this activator, so that if the vitamin were added *in vitro*, the activities might not get closer together?

DR. ZORZOLI: That is a possibility and I believe Schwachman and Gould made some experiments along that line and found there was no increase in the phosphatase activity. Vitamin C is not an activator of alkaline phosphatase *in vitro*.

DR. VICTOR EMMEL (*Rochester, N. Y.*): I quite agree with Dr. Glick about the desirability of trying to improve our method in every possible way. The point I want to emphasize is that there is a difference that should be investigated and not simply ignored.

DR. R. W. MOWRY (*Boston, Mass.*): With respect to Dr. Emmel's work, he raises the question that it would be adverse evidence if numerous agents caused such a relative blocking of renal phosphatase. Have you tried a number of other agents to see whether you get the same kind of difference? I ask the question because I have observed that formalin invariably seemed to have less effect on duodenal phosphatase than on renal phosphatase. The same result was obtained with bichromates and a number of other agents.

DR. EMMEL: I have observed the same thing with formalin, as has been reported by Dr. Gould, who studied rather highly purified preparations of phosphatase from the kidney and the intestine. He felt that intestinal phosphatase was less affected by formaldehyde. I believe that is borne out by histochemical studies.

Evaluation of the Histochemical Alkaline Phosphatase Techniques. H. O. YOKOYAMA, R. E. STOWELL, and K. K. TSUBOI, *University of Kansas, Kansas City, Kans.*

Abstract. Two histochemical alkaline phosphatase techniques, that of Gomori and Takamatsu and the newer Manheimer and Seligman method have been studied. Several organs of the mice were used for the following purposes: (1) Comparison of the cytological localization of the enzyme by the two techniques; (2) an evaluation of the localization of alkaline phosphatase by histochemical techniques; and (3) correlation of histochemical results with those obtained chemically on isolated cellular fractions.

There are noticeable differences in the enzyme activity of nuclei in tissue sections by the two histochemical techniques. Gomori's method shows a definite nuclear activity in many organs, whereas the newer method shows very little. Chemically, little or no alkaline phosphatase activity was found in mouse liver nuclei isolated by dilute citric acid as recommended by Dounce for the preservation of enzyme activity.

Gomori's technique shows a definite diffusion of the alkaline phosphatase from areas of higher concentration to lower concentration during the incubation procedure. This conclusion is based on the results of diverse studies on the possibility of enzyme diffusion.

Criteria of Validity in Enzymatic Histochemical Studies. J. S. FRIEDENWALD, *Johns Hopkins Hospital, Baltimore, Md.*

Abstract. Validity of conclusions from studies in enzymatic histochemistry rest (a) on the specificity of the reaction for the identification of the enzyme and (b) on the reliability of the localizing process. In respect to (a), very little attention has heretofore been given to the use of specific inhibitors and activators. In respect

to (b), problems arise through the formation of nonenzymatically generated precipitates, through diffusion of the enzyme and of the reaction product. Safeguards in respect to these difficulties will be suggested and their reliability discussed.

Discussion

DR. A. B. NOVIKOFF (*Burlington, Vt.*): I would like to comment on Dr. Yokoyama's paper. It is perhaps worth noting that independently, as far as I can tell, at least three laboratories in this country are working on practically the same problem and attacking it in essentially the same way: the validity of the Gomori phosphatase method as a cytochemical tool. We, like Yokoyama *et al.*, have been working with alkaline phosphatase. Dr. Palade at the Rockefeller Institute has been working with acid phosphatase.

When we isolated the cell particulates of rat liver, by the method of Hogeboom, Schneider *et al.*, much to our surprise, only some fifteen percent of the total alkaline phosphatase activity was in the nuclear fraction. The mitochondrial fraction had about the same percentage of the total activity; the microsomes had practically no activity; and about 60 percent of the activity was in the supernatant fluid.

Then we asked whether we were extracting the alkaline phosphatase from the nuclei in the process of fractionating the cells, or whether the histochemical method was misleading, staining the nuclei darkly desp[i]e the relative scarcity of enzyme inside the nuclei.

Dr. Yokoyama's paper has shown us how the nucleus can stain darkly even though it has no enzymatic activity. We have done essentially the same kinds of experiments and can confirm them in almost every detail.

I think it is worth commenting that we confirm the point of difference between Dr. Yokoyama and Danielli. You recall that Danielli emphasized that you get nonspecific adsorption in the very areas in which enzyme activity is high. We have a fine example to show that this is not so, at least not invariably. In normal rat liver, the regions of intense alkaline phosphatase activity are the areas surrounding the bile ducts. In the inactivated sections, incubated in substrate to which we added a bit of either purified alkaline phosphatase or supernatant fluid, you get beautiful staining in the nuclei, just as Yokoyama *et al.* have shown. But there is no staining at all around the bile ducts; these areas do not adsorb the calcium phosphate, despite their high enzyme activity.

DR. R. W. MOWRY (*Boston, Mass.*): I wonder if Dr. Friedenwald makes any allowance at all for the differences in solubility and the differences in phosphate molecules in the different phosphates you used.

DR. FRIEDENWALD: All the substrates were used at the same molarity.

DR. G. D. MAENGWYN-DAVIES: May I say that Jacoby and Martin in 1949 showed the diffusion in the liver slices from the overlying kidney slice, and also in embedded tissue.

Pathological Deposits of Calcium and Iron. HENRY BUNTING, *Yale University School of Medicine, New Haven, Conn.*

Abstract. A study has been conducted on pathological material containing zones commonly recognized in hematoxylin-eosin preparations as calcified. The composition of these deposits in respect to iron and calcium has been repeatedly debated by investigators, since their methods have lacked the necessary specificity. Included in the group are the systemic blood vessels and those of the central nervous system; the valve ring of the heart; interstitial deposits in the kidney, choroid plexus, and in the brain; and certain others to be described. Calcium has been identified by the formation with alizarin red S of orange lakes that have proved to be crystalline and birefringent, and by the formation of gypsum crystals; ferrous and ferric iron by HCl and ferri- and ferro-cyanide, respectively, with microincineration for confirmation;

inorganic phosphorus by a method slightly modified from Feigl's employing ammonium molybdate—HNO_3 and subsequently benzidine and sodium acetate; carbonate by applying acid and observing the production of gas bubbles that yield a precipitate with $Ba(OH)_2$.

The results based on 65 examples are as follows:

The deposits in the blood vessel walls have yielded calcium, ferrous and ferric iron, carbonate and phosphate in most instances; in a few either calcium or iron and carbonate have not been found. The deposits in the valve ring of the heart and in the interstitial tissues elsewhere have yielded in all instances calcium, ferrous and ferric iron, carbonate and phosphate. Necrotic myocardial fibers associated with renal insufficiency have been found to contain only identifiable iron and phosphate. In regions of marked hemolysis (as in the spleen in hemolytic jaundice) deposits of ferric and ferrous iron and phosphate are present in the interstitial tissues and in the elastica of the vessels. Iron of hemosiderin type in phagocytes has been found invariably to be ferric while iron in these interstitial deposits even in the fresh state is in most instances both ferric and ferrous and easily converted from one valence to the other by mild reagents; it is readily ionizable, at times reacting with ferrocyanide in the absence of HCl. The evidence shows clearly that ionized iron as well as calcium is present in appreciable quantities in various interstitial "calcified" deposits where they must be combined in some manner with acidic groups such as carbonate and phosphate. The mechanism of their deposition is not yet apparent, but there is no evidence of cellular participation.

Discussion

DR. W. W. AYRES (*Bethesda, Md.*): We have noticed certain laminated anisotropic crystal deposits in the kidney. With a little further study we find black deposits that we believe, as you have illustrated, indicative of iron deposition in calcium. It was my belief at the time that there is first a deposition of the calcium and later a deposition of the iron, which would explain the black staining with the hematoxylin. Has that been your experience?

DR. BUNTING: The stages of formation of such a deposit are very hard to reconstruct from observations as those, and I do not believe I know which comes first. In the kidney, as you know, Wachstein has described the participation of the alkaline phosphatase from necrotic proximal convoluted tubule epithelium as bringing about the calcification of these cell masses. In regions of bone formation iron can be demonstrated where the actual calcification is occurring (as in the subperiosteum). This has been demonstrated by Dr. W. G. MacCallum, who felt that the iron preceded the actual deposition of calcium. I do not believe that I have any definite evidence except perhaps from the necrotic myocardial fibers. In the example demonstrated there was only iron in combination apparently with phosphate, but we know that this may go on to actual calcification. The situation may well vary from spot to spot, and it is very hard to trace the preceding steps from the final process as observed.

DR. H. SHAPIRO (*Philadelphia, Pa.*): Can you think of any source of iron other than the hemoglobin, which would give the precipitate?

DR. BUNTING: That has been debated for a long time by many pathologists. The neurones do contain iron and in such instances as this, where there has been a dropping out of neurones as part of the senile process, the opinion has arisen that iron originated, in part at least, from the destroyed nerve cells; but I have no way of proving this.

DR. SHAPIRO: I don't know whether I should take the time to discuss this, but there is an interesting physiological observation that bears upon Dr. Bunting's slides, and also the work introduced by Dr. Drabkin. That has to do with the fact that cytochrome *c* does contain iron, and of course in cytochrome destruction in pathological processes you might have in consequence the appearance of the iron in the tissues. The precipitation of this iron might be expected to affect cellular metabolism, or processes dependent upon metabolism, such as rhythmical electrical potentials.

In his interesting studies on pacemakers in biological systems, Dr. Hudson Hoagland showed that when he measured the alpha frequency in the electroencephalogram of

normal people and varied their body temperature by means of diathermy, an Arrhenius mu value of 8,000 calories resulted. Comparing that with the value found from similar experiments with luetic patients (in which disease histological examination may reveal iron precipitated in the brain), he found a different temperature coefficient, *viz*, a mu value of 16,000 calories, indicative of an iron-containing link as the pacemaker reaction.

If cellular oxidations in the brain normally go through the cytochrome step in the chain of enzymatic processes, precipitation and destruction of this enzyme by pathological processes would minimize its active concentration in the cells, and result in its acting as a bottle neck, and thus becoming the pacemaker reaction.

DR. BUNTING: In luetic patients there is much hemosiderin iron in addition to the type described here.

DR. R. D. LILLIE (*Bethesda, Md.*): Dr. Bunting, do you consider that the von Kossa reaction, which is after all a phosphate reaction, could occur purely on the basis of phosphate present in the absence of calcium?

DR. BUNTING: Yes, the von Kossa reaction depends on the formation of insoluble salts of silver, that is, with carbonate or phosphate and perhaps others.

DR. LILLIE: And might be present purely in the presence of an iron deposit of this type in the absence of true calcium?

DR. BUNTING: Yes, indeed.

Adaptation of Bennett's Sulfhydryl Staining Technique for Use as a Rapid Histochemical and Quantitative Chemical Method. H. MESCON[1] and P. FLESCH, *University of Pennsylvania, Philadelphia, Pa.*

Abstract. The use of the Adamstone-Taylor frozen section technique enabled us to stain directly sulfhydryl compounds in fresh tissue sections with Bennett's sulfhydryl reagent [1-(4-chloromercuriphenylazo)-naphthol-2]. Immediately after removal from animals, the skin was placed on the stage of a freezing microtome and sectioned at 5–7 μ. The section was kept frozen, placed on a slide, and as it thawed was immediately immersed in a saturated 80 percent alcoholic solution of the reagent for 1 to 3 hours. The slides then were immersed for 1 minute in 95 percent alcohol, dehydrated in absolute alcohol, cleared in xylene, and finally mounted in balsam. Sections retained their color during a 3-month observation period. Autopsy and biopsy specimens of normal and pathologic (neoplastic, inflammatory . . .) human skin, and fresh skin specimens of mice and frogs were stained in this way. A colorimetric procedure for the quantitative estimation of sulfhydryl compounds was developed by using an amyl acetate solution of the reagent. The sulfhydryl content of 5 to 10 mg. of tissue could be determined in this way.

Discussion

DR. DAVID GLICK (*Minneapolis, Minn.*): Do I understand you correctly that you formed a precipitate with reagent, thinned it down, and then measured the color?

DR. MESCON: Dr. Flesch can tell you.

DR. PETER FLESCH (*Philadelphia, Pa.*): We removed the supernate. Since the color of the reagent is too weak for colorimetric estimation, we have to add a strong acid which intensifies the color.

READ BY TITLE

Histochemical Studies in Generalized and Circumscribed Myxedema. R. H. FOLLIS, *Johns Hopkins University Medical School, Baltimore, Md.*

Abstract. We have had occasion to study skin biopsies from patients with generalized myxedema associated with hypothyroidism and circumscribed (pretibial) myxedema

[1] This work was done in part under a Damon Runyon Clinical Research Fellowship recommended by the Committee on Growth of the National Research Council and administered by the American Cancer Society, Inc.

associated with hypothyroidism. A number of stains have been applied of which toluidine blue and the periodic acid-leucofuchsin reactions have been most interesting. The basophilic inter-collagenous material which is found in both types of myxedema is intensely metachromatic and gives a positive reaction with the Schiff reagent. The character of the changes in the collagenous fibers appears to be different in the two types of lesions. A number of enzymes have been applied both to these two types of myxedematous lesions as well as to normal skin. The results of these findings will be discussed.

Sources of Error in Enzymatic Histochemistry. G. GOMORI, *University of Chicago, Chicago, Ill.*

Abstract. Errors in histochemistry are discussed under three headings: false positive reactions, false negative reactions, and false localization. The last group of errors is the most important one; the cause is supersaturation of the incubating medium with products of hydrolysis, formed in the course of unduly prolonged incubation at nonoptimal pH levels. Specific instances of erroneous results and/or erroneous interpretation are given.

Histochemical Studies of Techniques for Mucopolysaccharides. R. E. MANCINI, *Inst. Noc. de Endocrinol., Buenos Aires, Argentina.*

Abstract. The following techniques were applied to several mucin-containing tissues: 1) metachromatic toluidine blue stain; 2) McManus' technique; 3) Hale's colloidal iron technique; 4) Mayer's mucicarmine; 5) photo-chemical reaction (technique of Etcheverry and Mancini). The latter is as follows: The sections are treated with an acid solution of sodium tungstate and then exposed to a short ultraviolet irradiation. A blue compound is formed which is not stable. The McManus' and photochemical techniques give a positive reaction with sulphurated mucins, hyaluronic acid, glycogen, starch, cellulose, and chitins. The metachromatic technique does so only with acid and sulphurated mucins. Hale's technique gives positive results with mucins and hyaluronates, and weakly positive ones with glycogen, starch, cellulose, and chitins. The mucicarmine is positive only when applied to sulphurated mucins,and in a lesser way when applied to hyaluronic acid, glycogen, and cellulose. Previous digestion of sections with hyaluronidase turned all these reactions negative, only in umbilical cord, synovial membrane, and vitreous body. In other tissues with a high content of epithelial or connective mucins, reversal of the reactions was either partial or did not occur at all.

Histochemical Study on the Action of Hyaluronidase Upon Fibroblasts Cultivated *in Vitro*. R. E. MANCINI and EUGENIA SACERDOTE DE LUSTIG, *Inst. Nac. de Endocrinol., Buenos Aires, Argentina.*

Abstract. Hyaluronidase was added to chick embryo fibroblast cultures both in hanging drop and Carrel flask. Swelling, progressive vacuolization, and disintegration of the fibroblasts were observed. These are reversible to a certain point.

In normal fibroblasts perinuclear and extracellular granules can be demonstrated with McManus' technique and with the toluidine blue metachromatic stain. Hale's colloidal iron technique did not give any positive result. These mucin granules almost completely disappear when hyaluronidase is added to the fibroblast cultures. These results suggest that a mucin, constituted mainly by hyaluronic acid, is formed by fibroblasts cultivated *in vitro*.

The swelling and vacuolization of fibroblasts can be explained by digestion of the intracellular mucin by hyaluronidase. Intermediate highly hydrophilic substances are then set free. The changes undergone by fibroblasts may be ascribed to these intermediate substances.

A Study of Intracellular Minerals and Mitochondria in Tumor Cells and in Dividing Nerve Cells. R. C. MacCardle, *National Institutes of Health, Bethesda, Md.*

Abstract. Intracellular minerals, revealed by microincineration and other histochemical methods, were observed in sarcoma cells and in young mitotically dividing nerve cells. It was found that the nucleus contained large quantities of calcium and/or magnesium ash, much of which was released to the cytoplasm upon rupture of the nucleus in mitosis. The mineral skeleton of the mitotic figure was sometimes moved *in toto* by ultracentrifugation. After treatment with mitotic inhibitors, iron apparently accumulated in the region of arrested chromosomes. There was no evidence of presence of iron in mitochondria. The mitochondria of sarcoma 37 were rod-shaped, filamentous, or globular. In tumors treated with mitotic inhibitors, the mitochondria of resting cells were rod-shaped, while in the arrested dividing cells, they were vesiculated. Bizarre mitotic figures appeared in nerve cells of the central nervous system in newborn mice after administration of mitotic inhibitors, in which most of the iron was found in the peripheral cytoplasm. In mature nerve cells, the mineral residue of neurofibrils contained iron.

Cytochemical Studies on the Intranuclear Inclusion of Herpes Simplex. Helen V. Crouse, L. L. Coriell, H. Blank, and T. F. McNair Scott, *University of Pennsylvania, Philadelphia, Pa.*

Abstract. Histochemical studies on the inclusion body of herpes simplex in the chorioallantois at various intervals after infection indicate that the formation of the inclusion is a changing dynamic process involving many stages which present different morphologic and tinctorial characteristics.

In the early inclusion a distinct accumulation of nucleoprotein of the desoxyribose type is present.

In the older inclusions presenting the "classical type A", nucleoprotein is no longer present within the nucleus.

An increase in protein synthesis within infected cells is suggested by the very large size of the nucleolus, and by increased alkaline phosphatase activity.

Use of Chemical Histology in the Analysis of Ovarian Activity. W. B. Atkinson[1] and P. Wright, *University of Montana, Missoula, Mont.*

Abstract. Implantation in the weasel is delayed from 4 to 6 months after mating. After implantation pregnancy lasts but 3 weeks. The role of the ovarian hormones is unknown.

The paucity of animals made it impossible to evaluate hormonal activity on the basis of uterine morphology alone. Since changes in chemical histology are frequently more pronounced than accompanying cytological changes, several cytoplasmic constituents were studied in the endometria of hormone-treated castrates. Ribonucleic acid, sudanophilic lipids, and lipase showed no distinctive patterns. On the other hand, progesterone produced a marked increase in alkaline phosphatase, whereas estrogen together with progesterone or androgen decreased phosphatase slightly as compared with the castrate. Although estrogen alone did not affect phosphatase, either alone or with androgen it produced a considerable increase in mucopolysaccharide in the glands. Progesterone did not affect mucopolysaccharides. The application of these histochemical observations to the analysis of ovarian activity in the pregnant animal will be discussed.

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[1] Now at Camp Detrick, Md.

Renal Alkaline Phosphatase in Diabetes Mellitus: A Histochemical Study. R. W. MOWRY,[1] *Mallory Institute of Pathology, Boston City Hospital, Boston, Mass.*

Abstract. The kidneys, next to the small intestines, contain the greatest concentration of alkaline phosphatase found in human tissues. The enzyme is localized almost solely to the proximal convoluted tubules, where its function is uncertain. One postulated role concerns the reabsorption of glucose from the glomerular filtrate, even though certain experimental evidence suggests that phosphorylase is of more importance in this process.

The present study was undertaken to determine if the human diabetic state is accompanied by any detectable alterations in alkaline phosphatase content or distribution. Blocks of kidneys were taken from both diabetics and controls (nondiabetics) at postmortem examination. These were fixed in alcohol, taken through petroleum ether, and embedded after brief paraffin infiltration. Enzyme activity was demonstrated in paraffin sections by Gomori's technique. In view of the variations in alkaline phosphatase activity and pattern known to occur in certain renal diseases, the controls include both normal and abnormal kidneys.

It is tentatively concluded that discernible alterations of alkaline phosphatase do not occur in the kidneys of diabetics unless complicated by renal damage.

Some Histochemical Reactions of Teeth and Bone. R. O. GREEP, *Harvard School of Dental Medicine, Boston, Mass.*

Abstract. The localization and distribution of some histochemically reactive substances have been studied in the hard structures of rats. Changes were induced in the rate of matrix formation or in mineralization by experimental procedures. The concentration of alkaline phosphatase is unusually heavy in cells bordering on or subjacent to areas of active matrix formation, irrespective of the deposition of minerals. This indicates, although inconclusively, that alkaline phosphatase is important in the formation of the ground substances. This need not prejudice its possible role in the deposition of phosphate-bearing salts.

Basophilia and metachromasia in the incisors, mandibular condyle, costochondral junction and proximal end of the tibia as observed under these different growth states will be discussed.

The Effect of Storage and Stilbamidine on the Nucleic Acid Content and Virulence of Mouse Ascites Tumor. N. B. KURNICK, *Tulane University, New Orleans, La.*

Abstract. Ehrlich carcinoma, inoculated intraperitoneally into mice, produces ascites loaded with free floating tumor cells. This fluid may be aspirated, stored at 4° C for some time, and reinoculated with production of the ascites carcinoma. Cell-free fluids or fluids in which the cells have been mechanically disrupted are avirulent. It is found that fluids stored for 9 days lose virulence (measured by prolonged incubation period and survival time at a given cell dosage level). This loss in virulence is associated with loss in ribonucleic acid content (50–80 per cent) but not in desoxyribonucleic acid content or cell number. Histologically, there is a marked, general loss in cytoplasmic absorption at 2600 Å. Viability was confirmed by cinematography. Storage at 37° C for 24 hours results in death of the cells and complete loss of virulence.

Stilbamidine, both *in vivo* and *in vitro* produces cytoplasmic inclusions consisting of stilbamidine ribonucleate in the cancer cells. However, no loss of virulence results. There appears to be a compensatory increase in ribonucleic acid content *in vivo*. Cells treated with stilbamidine appear to be more sensitive to storage in the cold than untreated cells, since 24 hours' storage produces significant loss of virulence in stilbamidinized cells, suggesting that if ribonucleic acid "replacement" is interfered with, cell injury results.

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[1] S. A. Surgeon, U. S. Public Health Service, National Institutes of Health, E. B. M. I.

Behavior of the Plasma Membrane and Cell Wall of *Bacillus* Cells in Certain Staining Procedures. C. F. Robinow, *University of Western Ontario, London, Canada.*

Abstract. The dyes most widely used in the staining of bacteria, *e. g.* crystal violet, basic fuchsin, or methylene blue, have more affinity for the interior of the bacterial protoplast than for the material composing its surface and pass right through the plasma membrane without staining it. This is not apparent when bacteria are fixed by heat and heavily overstained in the customary manner, but is easily seen when very dilute dye solutions are applied to bacteria that have been fixed in the wet state. Victoria blue (4R and B) behaves in the opposite way and is arrested in the plasma membrane. In this way two very different and complementary aspects of the cell are obtained.

In the Dyar method for the staining of the bacterial cell wall, a colored deposit is formed on, and possibly in, the cell wall. This occurs when a cationic detergent, diffusing outward through the cell wall meets with an excess of the dye Congo red in the surrounding fluid. The deposit is reminiscent of myelin figures and is birefringent and dichroic. The configuration of the deposit is consistently different in different parts of the cell. The procedure provides an indirect means of studying the structure of the cell wall.

Alteration of Ribonucleic Acid Distribution in the Liver. P. B. Szanto and H. Popper, *Hektoen Institute, Cook County Hospital, Chicago, Ill.*

Abstract. The alterations of the distribution of cytoplasmic and nucleolar ribonucleic acid were studied in human livers obtained by necropsy and biopsy as well as in livers of animals subjected to various types of hepatic injury. The ribonucleic acid was identified mainly by staining with various dyes controlled by specific enzymatic digestion and to some extent also by fluorescence microscopy. The alterations of the distribution in hepatic cells including a decrease appear characteristic of certain aberrations of these cells. An increase in Kupffer cells and histiocytes indicates stimulation of hepatic mesenchyma. Together the alterations are diagnostic for various hepatic diseases. The histochemical findings were also related to the serum proteins in that in the human as a general rule, elevation of serum globulin coincided with increase of ribonucleic acid in the mesenchymal elements and a decrease in the hepatic cells with reduction of serum albumin.

Disturbances in Nucleic Acid Metabolism Produced by X and Gamma Radiations in Human Malignant Tumors. L. Hamilton, *University of Cambridge, England; now at University of Utah, Salt Lake City, Utah.*

Abstract. A series of sections from biopsies of 86 cases of human malignant tumors, taken before and at one or more intervals following irradiation, received identical histological and cytochemical treatments. These included the use of a modified Brachet's test and the Feulgen reaction. Changes in the basophilia of the cytoplasm in each series were graded, so that every experimental result was classified as +, o, and −, *i. e.* increase, no change, or decrease. Doubtful results were assigned to the zero class. These results were obtained without prior knowledge of the quantity, type, duration of irradiation, or of the period after irradiation at which the biopsy was taken.

Each case was classified under several heads, (a) type of irradiation, X or γ, (b) total dose, (c) dose rate, (d) time after completion of irradiation when biopsies were taken, (e) duration of treatment, and the correlation between these factors and the changes in the cytoplasmic basophilia was determined. The most significant correlation was that with the total dose. With doses up to 300r there is an increase, between 300r to 1000r there is either an increase or no change, and over 1000r a decrease in the ribonucleic acid content of the cytoplasm of the irradiated tumor cells. There was little or no correlation with the other factors listed above.

An Attempt to Produce Structural Changes in Nerve Cells by Intense Functional Excitation Induced Electrically. CHAN-NAO LIU, *University of Pennsylvania, Philadelphia, Pa.*

Abstract. Experiments were performed in guinea pigs and cats to determine whether or not structural changes in nerve cells could be produced by electrically induced activity in neurons. Comparison of sections of control and experimental guinea pigs failed to reveal any change of Nissl pattern in the cells of spinal ganglia and spinal cords after prolonged electrical stimulation when adequate precautions against post-mortem and fixation artifacts were observed.

Prolonged antidromic stimulation of the cat's hypoglossal nucleus failed to produce detectable change with stimuli having intensities below 20 volts. With intensities of 20 volts or greater, changes were observed 60 hours after stimulation. These changes resembled early stages of chromatolysis, suggesting damage to fibers and subsequent axon reaction in the cell cytoplasm. Clamping the hypoglossal nerve resulted 60 hours later in less marked changes of a somewhat similar type.

Stimulation of the hypoglossal nerve for short intervals induced no changes that could be detected by staining of nerve cells or by ultraviolet photography in the 2537 Å light band.

A New Method for the Demonstration of Spirochetes. E. D. DELAMATER, *University, of Pennsylvania, Philadelphia, Pa.*

Abstract. A new stain for the demonstration of spirochetes has been elaborated that appears to be the first method for the demonstration and study of these organisms that gives a high degree of specificity and visualization. Previous methods have been both inconsistent and questionable in their specificity. The present method depends upon a double mordanted process, and the question was raised as to the point of action of these mordants that makes this stain possible. Although highly selective, the stain does not appear to be chemically specific.

Microspectrophotometric Studies of Some Histochemical Reactions. C. R. HOOVER and L. E. THOMAS, *University of Missouri, Columbia, Mo.*

Abstract. The Feulgen and arginine histochemical reactions have been tested microspectrophotometrically for conformity with the Beer-Lambert law. For this purpose nucleoprotein was incorporated into agar blocks which were then fixed, dehydrated, embedded, and sectioned in the manner usually employed for tissues.

Feulgen treated material was found to have different light absorption characteristics with Bouin's fixation as compared to Carnoy's (3:1) fixation.

The ratio of the light extinction of Feulgen-treated material to the extinction of material treated by the arginine method was determined on the agar-protein sections and on nuclei of a few tissues. This extinction ratio is interpreted in terms of possible type and distribution of protein in nuclei.

The Mast Cell Response to Administration of a Bacterial Pyrogen.[1] E. G. STUART, *University of Pennsylvania, Philadelphia, Pa.*

Abstract. The connective tissue mast cells in the abdominal skin of the mouse were studied by the technique of Zahl and Nowak (*Stain Tech.*, vol. 24, July, 1949). In normal animals they are numerous along capillaries and small venules. Elsewhere in the connective tissue they occur in scattered groups. Their granules are typically concentrated about a central nucleus so that the cells have a round or oval appearance. A few have indefinite cell boundaries due to dispersion of the granules.

A highly purified extract prepared from a pseudomonas species (Baxter Laboratories "Pyromen") was administered intraperitoneally and orally. Dosages varied from 5 μg./kg. to 50 μg./kg. Skin biopsies were taken at intervals varying from 15 minutes

[1] Aided by a grant from Baxter Laboratories, Inc., Morton Grove, Ill.

to 24 hours. After Pyromen administration the granules of many mast cells began to disperse and disappeared. In the immediate vicinity of the small vessels the dispersion began between 2 and 15 minutes after administering the drug, depending upon concentration of dosage. The groups of mast cells lying some distance from blood vessels were affected less rapidly. The shape of the mast cells changed; they became less discretely ovoid, their granules spread out widely away from the cell center and many of the cells along the capillaries became spindle shaped.

Perinuclear Sudanophilic Bodies in Mammalian Epidermal Cells. WILLIAM MONTAGNA, *Arnold Biological Laboratory, Brown University, Providence, R. I.*

Abstract. The epidermis of skin fixed in formalin or formol-calcium, when treated with Sudan black, attains a vague blue-grey color. When fixed according to the acid hematein routine of Baker ('46), the epidermal cells contain discrete perinuclear sudanophilic bodies, in the form of single or clustered spherules; occasionally they resemble the myelin figures of Palade and Claude ('49). The smaller sudanophilic globules are blackened solidly by Sudan, but the larger ones possess a sudanophobic core. While some of the sudanophobic centers are barely visible, others are large (2–3 μ) and are surrounded by delicate sudanophilic squamae which resemble the lepidosome of Parat ('26) and Baker ('49). Most cells have a cluster of larger vacuolated sudanophilic bodies at one pole of the cytoplasm and single smaller spherules around the nucleus. The structural plan of these sudanophilic bodies is similar to that of the Golgi bodies recently described by Baker ('49) in the spermatocytes of *Helix*. These elements can be seen in unstained preparations under phase-contrast. They can be revealed, although less clearly, with oil-blue N and Nile blue sulfate in properly fixed tissues. They resemble those in osmic preparations of similar tissues, and since they have a remarkable similarity to the Golgi bodies of Baker and his associates, it is likely that they represent the lipid component of the Golgi element.

The Presence of Periodic-Reactive Carbohydrates in the Thymus Glands of Mice.[1] CHRISTIANNA SMITH, *Mount Holyoke College, South Hadley, Mass.*

Abstract. The thymus glands of a series of mice from a fetal length of 10 mm. to animals 700 days of age were fixed in ice-cold Rossman's fluid and stained by the periodic acid-leucofuchsin technique of McManus ('48). In the fetal thymus the cells containing glycogen were particularly prominent at the anterior pole and in a more or less complete peripheral zone. Most of the cells containing glycogen were vacuolated epithelial cells. Some lymphocytes containing glycogen were seen in the thymus glands of mice just before birth. In the newborn and until 14 days of age about one-third of the cortical lymphocytes were found with glycogen in them (Smith and Thomas '50). After this time the glycogen in the lymphocytes gradually disappeared. In the old thymus glands where intrathymic fat cells were developing, glycogen was found. In the involuting thymus glands Russell's body cells, foamy cells, and mast cells were strongly colored in both undigested and digested sections. In addition to the study of the reaction of the different cells to the periodic acid-leucofuchsin technique during the life history of the thymus gland, the differentiation of the reticular fibers in the fetal thymus gland also is under investigation.

Histochemical Study of the Renal Lesions of Experimental Diabetes. R. E. MANCINI, *Inst. Nac. de Endocrinol., Buenos Aires, Argentina.*

Abstract. The changes of the glomerulus and renal tubule were studied in rats with diabetes brought about by sub-total (95%) pancreatectomy. During the initial

[1] Aided in part by a grant from the Public Health Service.

stages (2 to 4 months) of the disease, the McManus HIO_4-Schiff technique gives a very marked positive reaction in the visceral basal membrane and in the "mesangium" of the glomeruli. On the other hand, the metachromatic technique gave a negative reaction. Digestion of the sections with hyaluronidase showed that this substance is not hyaluronic acid. In the final stages of diabetes, together with the development of a glomerular sclerosis formed by reticulum fibrils, the McManus reaction becomes much weaker in the basal membranes and in the "mesangium."

In the proximal convoluted tubule, the alkaline phosphatase and the McManus reaction, which stains the brush border of the cells, lose intensity progressively as diabetes goes on, until they disappear almost completely in the final stage of the disease. The lipids of the renal tubule suffer no remarkable changes. On the other hand, in the final stage of diabetes, the occurrence of glycogen in the loop of Henle and in the distal convoluted tubule and collector tubule is constant.

Histochemical Study of the Toad Testicle and Its Relationships With the Hypophysis. R. E. Mancini and M. H. Burgos, *Inst. Nac. de Endocrinol., Buenos Aires, Argentina.*

Abstract. An histological and histochemical study was made in the toad testicle under the following conditions: 1) seasonal changes throughout the year; 2) under the action of gonadotropins; 3) following hypophysectomy.

A continuous annual spermatogenic cycle exists. In spring, spermatogonia predominate; in summer and autumn, spermatocytes I and spermatids; in winter, spermatozoa predominate with an increase till spring as a preparation to sexual intercourse.

The spermatogenic line lacks glycogen, lipids, and peroxidases. The nuclei of spermatocytes I, spermatids and spermatozoa heads contain an alkaline phosphatase. A mucopolysaccharide was shown to appear at the same time as the intercellular vacuolization of spermatids during their displacement towards the Sertoli cells. In the interstitial cells there are lipids partly represented by steroids, as well as small amounts of ascorbic acid.

During the spring sexual intercourse, or following gonadotropin injection, the Sertoli cell becomes vacuolized and sets the spermatozoa free, and at the same time mucin, which was not present in this cell during the resting phase, appears. The prompt action of this hormone does not seem to modify the other histochemical components.

Hypophysectomy brings about in 2 months atrophy of the spermatogenic line and interstitium as evidenced by spermatocyte disorganization, nuclear pyknosis, signs of involution and disappearance of the mucin and phosphatase. The lipid and ascorbic acid content of the interstitial cells diminishes as well.

The following techniques were applied: hematoxylin eosin, McManus' HIO_4-Schiff, a metachromatic stain, a photochemical reaction for mucin, the iodine reaction for glycogen, Sudan III and Sudan black, Schiff's and Schultz's reactions and birefringence for lipids, and Gomori's technique for alkaline phosphatase. Ascorbic acid was determined with Deane's and Morse's modifications of the original technique.

Carbonyl Lipid Reactions in the Adrenal Gland of the Rat, With Special Reference to the Acetals. E. R. Hayes, *Ohio State University, Columbus, Ohio.*

Abstract. The plasmal reaction, as recently re-defined, demonstrates only acetal lipids. Histochemical findings on the adrenal gland of the rat indicate that this reaction is not the same as that obtained after formol fixation and/or oxidation (Bennett reaction). Material positive to the latter reaction cannot be proved to be derived from acetals. The mechanism of both reactions is discussed. In a series of histochemical studies of the rat adrenal, the distribution of acetal lipids was shown to be unaffected by variations in the total lipid (Sudan black B) and cholesterol (Schultz)

pictures. Three types of lipid pictures in this organ were compared: (1) adrenal glands with normal amount and distribution of total lipid and cholesterol (normal adult males), (2) with depletion of total lipid and cholesterol in the zona fasciculata (cold, tourniquet shock, stilbestrol) and (3) with depletion in the zona glomerulosa (desoxycorticosterone acetate). The plasmal reaction was the same in all 3 groups. Possible reasons for the divergent results of various workers studying carbonyl lipids are suggested.

Histochemical Study of Leukocyte Glycogen Content Under Normal and Pathological Conditions. R. E. MANCINI, *Inst. Nac. de Endocrinol., Buenos Aires, Argentina.*

Abstract. The following techniques were applied to blood smears: iodine reaction (author's variant), Bauer's chromic acid, sulfite-leucofuchsin reaction, McManus' periodic acid, sulfite-leucofuchsin technique, and photochemical reaction (author's technique). Best results were obtained with the iodine reaction and with McManus' technique. With the iodine reaction the cytological variations of glycogen content in blood smears were better appreciated.

With Bauer's reaction and with iodine, glycogen was found only in neutrophils and metamyelocytes; this glycogen was digested by ptyalin.

With McManus' technique and with the photochemical reaction, glycogen was found in eosinophils as well. Sometimes these two reactions were positive in monocytes and lymphocytes, but this glycogen was not always digested by ptyalin. With the iodine reaction an increase in glycogen content was found in blood neutrophils in human diabetes, in experimental dog and rat diabetes, in infectious diseases, in acute myeloid leukemias, and in severe trauma and burns. The greatest increase was observed in neutrophils of serous and purulent exudates and in old transudates.

The Lipid Material of Hyaline Arteriolosclerosis. R. D. BAKER, and S. KENT, *Medical College of Alabama, Birmingham, Ala.*

Abstract. The lipid part (or at least the non-bound lipid) of hyaline renal arteriolosclerosis absorbs sudanophilic dyes readily (Sudan IV, Sudan black, and Oil-red-O) in unfixed or formalin-fixed frozen sections.

The Schultz modification of the Liebermann-Burchardt method for sterols gives the characteristic greenish reaction, suggesting a considerable cholesterol ester content of the hyaline regions of renal arteriolosclerosis; and the Marchi method, using osmium tetroxide after mordanting with potassium bichromate, is usually positive.

Solubility tests indicate that sudanophilic lipid is dissolved from unfixed frozen sections by acetone and absolute alcohol at room temperature in as short a time as 1 minute, while it is not dissolved by petroleum ether at the end of 24 hours. Benzene and chloroform often dissolve the lipid completely in 24 hours. The rapid solvent action of acetone and absolute alcohol was attributed to their property of miscibility with the aqueous components of the tissues.

Formalin-fixed hyaline arterioles of kidney are a trifle more tenacious of the lipid than the unfixed arterioles, when treated with solvents.

The diffuse lipid of atheromas of the aorta has staining reactions and solubilities similar to the lipid of arteriolosclerosis. The lipid in macrophages of atheromas is less readily dissolved by fat-solvents than the diffuse lipid.

The staining reactions and solubilities point to similarities between renal arteriolosclerosis and atheromatosis of the aorta. The close association of hypertension and renal arteriolosclerosis suggest, therefore, that essential hypertension may have the same cause as atherosclerosis of the larger arteries.

Peroxidase Reaction for Fresh Tissues Applied to Hypo- and Hyper-Functioning Thyroid Glands. R. E. MANCINI and M. F. VILLAMIL, *Inst. Nac. de Endocrinol., Buenos Aires, Argentina.*

Abstract. A new technique for the study of peroxidases in frozen sections of fresh tissues, based on the benzidine reaction, is proposed. Ammonium molybdate was used as a catalyzer, followed by picric acid fixation. In the thyroid glands of normal rats and guinea pigs, the reaction appeared as perinuclear granules in the follicular epithelium. In thyrotropin-treated guinea pigs, the reaction became more intense and spread over a large number of follicles. Hypophysectomy in the rat leads to disappearance of the reaction; treatment with pituitary extract prevents this effect. The most outstanding effect on this benzidine reaction in thiouracil- and iodide-treated animals was enhancement of the focal appearance, while the zones between active foci became negative. In *in vitro* incubated thyroid glands the reaction was not altered by the addition of thyroxine or sodium iodide to the medium; thiouracil inhibited the reaction when added at very high concentrations.

In human toxic diffuse goiters the reaction is intense and widespread. In nodular goiters the reaction is mild or moderate, and intense in perinodular tissue. Within the nodule, the reaction is intense and widespread in toxic goiters, and mild or negative in colloid nodules.

Histochemical Differentiation of Substrate Specific Alkaline Phosphatases in the Tissues of the Rat. GERTRUDE D. MAENGWYN-DAVIES and J. S. FRIEDENWALD, *Johns Hopkins University, Baltimore, Md.*

Abstract. In order to study the tissue localization of various substrate specific alkaline phosphatases, we have applied an adaptation of Gomori's technique (*Proc. Soc. Exp. Biol. & Med.* 42 : 21, 1939) to fresh frozen tissue sections. In such sections many enzymes tend to diffuse. Good localization was obtained when working with a substrate solution half saturated for sodium acetate, and fully saturated for calcium phosphate. After incubation the acetate was washed out with a water-alcohol-calcium chloride mixture, and the enzymatically deposited calcium phosphate converted to cobalt sulfide.

These studies were undertaken with the following substrates: glycerophosphate, glucose-1-phosphate, fructose-6-phosphate, hexose-diphosphate, calcium-β-napthyl-phosphate, and muscle adenosine-triphosphate. All substrates were employed at the same molarity. Evidence for the substrate specificity of a number of alkaline phosphatases will be presented on the basis of the effects of different inhibitors and activators, and of variations in diffusibility and organ distribution.

Needle Biopsy of the Liver. V. Observations on the Distribution of Alkaline Phosphatase and Its Diagnostic Significance. F. P. CLEVELAND, D. RICHFIELD, E. A. GALL, and L. SCHIFF, *University of Cincinnati, Cincinnati, Ohio.*

Abstract. The distribution of alkaline phosphatase in 112 liver biopsies was studied in the Gastric Laboratory of the Cincinnati General Hospital. The liver specimens were examined in a routine manner with hematoxylin and eosin and with a modification of the Gomori histochemical technique. The 112 cases were grouped according to the various lesions encountered. These were histologically normal livers, diffuse hepatitis, portal cirrhosis, fatty livers, obstructive bile stasis, malignant tumor, and miscellaneous lesions. The distribution of alkaline phosphatase granules in each of the groups is described. The results of this study are compared with those of other workers in this field of research. It is concluded from the results of this work that the determination of alkaline phosphatase content or location is of little or no value in the distinction between obstructive jaundice and hepatogenous jaundice. It is to be noted that the amount of alkaline phosphatase is increased in various disorders of the liver.

Formation of Tyrosine Crystals From Leukocytes With a Synthetic Detergent: Lack of Relationship to Malignant Disease. W. W. AYRES, *National Naval Medical Center, Bethesda, Md.*

Abstract. Buffy coat from blood was mixed with a synthetic detergent, dihexyl sodium sulfosuccinate (Aerosol MA). This resulted in the formation of tyrosine crystals. The blood of 44 patients with proved malignant disease showed tyrosine crystals in 54 percent of the cases; the blood of 33 patients with destructive, but nonneoplastic diseases showed crystals in 57 percent of the cases; the blood of 54 percent of 80 normal persons showed tyrosine crystals. Control studies indicated that the tyrosine crystals were derived from the leukocytes and not from plasma or red blood cells. It was also determined that tyrosine crystals would form from buffy coat, without the addition of a synthetic detergent, but only at a much later time, and not in as many instances. The addition of a synthetic detergent to malignant, and nonneoplastic tissue resulted in some instances in the formation of tyrosine and other types of crystals of unknown nature.

Oddities of Staining With Proteose-Silver. H. A. DAVENPORT and R. W. PORTER, *Northwestern University, Chicago, Ill.*

Abstract. The variability of argyrophilia seen in different tissue components is influenced by the manner of fixation of the tissue, by post-fixation treatment, and the nature of the staining solution used. The variables which affect the staining solution are its pH and rH while it is acting on the tissue and especially the inherent nature of the silver compound as determined by the method of preparing it. Tentative conclusions are as follows: (1) Most proteose-peptone silver precipitates redissolve in an excess of a concentrated proteose-peptone solution. The solutions which are stable on dilution can be used for staining. (2) Acetylation (blocking exposed amino radicals) of the proteose-peptone before combining it with silver has been the most consistently effective method of enhancing the subsequent staining of axis cylinders. (3) Methylation (blocking exposed carboxyls) gave a silver derivative which stained mucin of intestinal mucosa and endoneurial connective tissue with a high degree of intensity. (4) Control of pH of the proteose-silver-nitrate reaction (by addition of NaOH or Na-acetate) is essential for securing uuiformity of the resulting proteose-silver compound. (5) Stains of axis cylinder in a proteose-silver solution whose pH was about 4.0 were obtained, thus dispelling an earlier belief that such staining required a medium of pH 7.0 to 8.5. The manifestations of selectivities for structures other than neural ones indicate that silver staining will have extensive application, but much needs to be learned about the mechanism of its specificity.

Structural Changes Produced in Brown-Pearce Carcinoma Cells by Means of a Specific Antibody. B. KALFAYAN and J. G. KIDD, *New York Hospital, Cornell Medical Center, New York, N. Y.*

Abstract. Brown-Pearce carcinoma cells lose their viability when incubated with the antibody that reacts specifically with a distinctive constituent of them (*J. Exp. Med.*, 1946, *83:* 227–250). Furthermore, contrary to previous observations, they regularly manifest characteristic structural changes, evident both in fresh and in fixed preparations.

When the carcinoma cells are suspended as individuals in isotonic saline solution and incubated at 37° C with rabbit serum containing the specific antibody and complement, their cytoplasm immediately begins to swell and the cells become spherical. The cytoplasmic basophilism rapidly diminishes and within 10–15 minutes but little remains. Soon thereafter the cytoplasm in the fixed and stained preparations appears empty except for a moderate amount of amorphous acidophilic material together with a few rod-like, granular, and vesicular bodies; in fresh cells the formed materials appear to be suspended irregularly in a transparent fluid that distends the cytoplasm.

Meanwhile, the plasma membrane is converted into an irregularly thickened acidophilic structure with swollen cytoplasmic particles adherent to it; "blisters" do not develop from its outer surface as they regularly do within a few minutes from that of the viable cells suspended in saline or in rabbit serum devoid of antibody. The nuclei often remain intact for some time after the cytoplasmic changes have become advanced, but within 30–60 minutes they and their nucleoli begin to shrink and the nuclear membrane becomes wrinkled, though the chromatin often remains unchanged in appearance for several hours before the nuclei finally become pyknotic.

These alterations do not develop spontaneously while the cells remain for long periods in saline solution or in normal rabbit serum, and they have not been reproduced by any of several other means used experimentally.

INDEX OF AUTHORS

VOLUME 10

JOURNAL OF THE NATIONAL CANCER INSTITUTE

INDEX OF AUTHORS

Page

Aegerter, Ernest A. 255
Algire, Glenn H. 225*, 565
Andervont, Howard B. 193*, 201*, 405*, 895*, 927*, 1157*
Ayres, William W. 1239*
Baker, C. G. 1191*
Barrett, Morris K. 61, 81*
Bennett, J. G. 359
Bennison, B. E. 175*, 989*
Berenblum, I. 167*
Bierman, Howard R. 93*, 279*, 843
Bigelow, R. 1115
Bischoff, Fritz 67
Blackburn, Charles M. 337*
Boyle, Peter J. 179
Bryan, W. Ray 647*, 1215*
Bryant, Henry C. 459*
Burgoyne, F. H. 665*
Butt, Hugh R. 497
Byron, Ralph L., Jr 331
Carroll, Dorothy M. 657, 1199
Cauldwell, Earl W. 131
Changus, George W. 481*
Chipps, H. Davis 439*
Cliffton, Eugene E. 719*
Code, Charles F. 337
Comfort, Mandred W. 497
Copeland, D. H. 361
Cornman, I. 1123*
Cox, Alvin 523*
Crabb, Edward D. 581*
Craig, William R. 459
Cromer, Horace E. 497*
Dalton, A. J. 53, 815
Davenport, Horace W. 315*
Deringer, Margaret K. 119, 1139
Downing, V. 1273
Dubnik, Celia S. 815*, 1201
Duchesne, Emily 291
Duffy, Benedict J. 489
Dunlap, Charles E. 481
Dunn, Thelma B. 179, 725*, 895, 1157
Duryee, William R. 735*
Earle, Wilton R. 291*, 865*, 883, 943*, 1067*, 1105*
Edward, Mary-Frances 291
Evans, Virginia J. 291, 883, 943
Fishman, W. H. 1115*
Fodor, Paul J. 271
Fones, William S. 663*
Friesen, Stanley R. 545*

*Indicates senior authorship of the article appearing on the page designated.

	Page
Gaviser, David	443
Glick, David	321*, 993
Goldfischer, Rhoda L	339
Greenspan, Ezra M	657, 1199, 1295*
Greenstein, Jesse P	271*
Gruenstein, Margot	255
Hanawalt, Charlotte	67
Hare, W. V	359
Hartwell, J. L	665, 1273
Hebbel, Robert	505*
Heller, John R	299*
Hertz, Roy	61*
Heston, W. E	53, 119*, 125*, 665, 1139*
Hogeboom, G. H	969, 977, 983*
Hollander, Franklin	339*
Homburger, F	489*
Howes, Edward L	377*
Hrenoff, Michael K	93
Hubbard, Thomas B	303*, 443
Jedeloo, G. C	809*
Jenkins, R	843
Johnson, James M	1201
Kaplan, Henry S	267*, 407*
Kelsall, Margaret A	625*
King, Lester S	131*, 689*
Kiriluk, L. B	993*
Komarow, S. A	255
Kremen, A. J	993
Law, L. W	179*
Legallais, Frances Y	225
Leiter, J	1273*, 1295
Leuthardt, Florence M	271
Levillain, W. D	1139
Levine, Michael	1005*
Lignac, G. O. E	809
Ligtenberg, A. J	809
Lorenz, Egon	147, 355*, 359, 1215
Maver, Mary E	647
McClelland, James N	597
Meister, Alton	75*, 1191, 1263*
Meissner, William A	533*
Melroy, M. B	1, 11*
Moloney, John B	647, 1215
Moore, G. E	303
Morgan, Russell H	455
Morgan, Walter C	81
Morris, Harold P	815, 1201*
Morrow, Andrew G	657*, 1199*
Morrow, P. L	843*
Mostofi, F. K	989
Mühlbock, O	861*, 1259*
Nadel, E	1043*
Patterson, Cecil O	477*
Petermann, Mary L	489
Pollard, H. Marvin	459

*Indicates senior authorship of the article appearing on the page designated.

	Page
Potor, Aurelia	489
Prehn, R. T	893*
Rhoads, C. P	489
Richardson, Howard L	467*
Roach, John F	455*
Robertson, William Van B	53*
Rose, John E	605*
Rosenthal, Sanford M	89*
Ross, H. E	977
Sack, Theodore	105
Salmon, W. D	361*
Scharrer, Berta	375*
Schilling, E. L	865, 943, 883*, 1067, 1105
Schneider, W. C	969*, 977*, 983
Seligman, Arnold M	105*
Sells, Marguerite T	647, 1215
Shacter, Bernard	331*, 637*
Shay, Harry	255*
Shear, M. J	1273, 1295
Shelton, E	865, 1067, 1105
Shimkin, Michael B	331, 597*, 637
Sloan, Robert D	455
Smith, Fern	423*
Sokolow, Maurice	279
Spencer, R. R	1*, 11
State, David	443*
Steiner, Paul E	429*
Stewart, Harold L	147*, 359*, 665
Strait, Louis A	93
Strong, Leonell C	423
Swain, Robert W	605
Symeonidis, Alexander	711*
Thomas, Mary Ann	647, 1215
Thompson, J. W	29
Trunnell, Jack B	489
Tullner, William W	61
van Thiel, P. H	809
Voegtlin, Carl	29*
Wangensteen, Owen H	443
Warner, Douglas	67*
Westfall, Benton B	61
White, Julius	663
Young, Nelson F	489
Youngner, J. S	565*

* Indicates senior authorship of the article appearing on the page designated.

INDEX OF SUBJECTS

VOLUME 10

JOURNAL OF THE NATIONAL CANCER INSTITUTE

INDEX OF SUBJECTS

Page

Abstracts 215, 797, 1001

Acidity, gastric, in adenomatous gastric polyp. (Cramer, Comfort, Butt, and Berkson) *October* 497

Acid phosphatase in gastric content of patients with carcinoma of stomach. (Changus and Dunlap) *October* 481

C-Acylase and lactonase activity of hepatoma. (Meister) *August* 75

Address of welcome, fourth conference on gastric cancer. (Heller) *August* 299

Alpha-peltatin, beta-peltatin, and podophyllotoxin, effect on lymphomas and other transplanted tumors. (Greenspan, Leiter, and Shear) *June* 1295

——— Damage induced in Sarcoma 37 with, (Leiter, Downing, Hartwell, and Shear) *June* 1273

o-Aminoazotoluene, induction of hemangioendothelioma and sarcoma in mice with, (Andervont) *February* 927

Amphibian cell nuclei, the nature of radiation injury to, (Duryee) *December* 735

Announcements 221, 223, 805, 1003

Antiproteolytic reaction, iodoacetate index, and least coagulable protein reactions in relation to malignant neoplasia. (Cliffton) *December* 719

Ascorbic acid deficiency, changes in transplanted fibrosarcoma associated with, (Robertson, Dalton, and Heston) *August* 53

Bacterial species, principles involved in survival, unicellular species. (Melroy and Spencer) *August* 11

Beta-peltatin, damage induced in Sarcoma 37. (Leiter, Downing, Hartwell, and Shear) *June* 1273

——— Effect on lymphomas and other transplanted tumors. (Greenspan, Leiter, and Shear) *June* 1295

Biopsy of gastric tumor, use of esophagoscope for obtaining, (Patterson) *October* 477

Biotherapeutic action of *Trypanosoma cruzi* on tar carcinoma in mice. (Jedeloo, Lignac, Ligtenberg, and van Thiel) *February* 809

Biotin, dietary, influence in growth of malignant and nonmalignant tissues in rat bearing Hepatoma 31. (Voegtlin and Thompson) *August* 29

Blood, effect of ultrasonic vibration on normal and leukemic subjects. Morrow, Bierman, and Jenkins) *February* 843

Blood serum, human, catecholase and catecholase inhibitor activity. (Shacter and Shimkin) *December* 637

Borate buffer, comparative stability of agent of Chicken Tumor I in, (Bryan, Maver, Moloney, Thomas, and Sells) *December* 647

Breast, adenocarcinoma of, in rat following gastric instillation of methylcholanthrene. (Shay, Aegerter, Gruenstein, and Komarow) *October* 255

——— Attempt to detect tumor agent in strain C mice by x-radiation. (Andervont and Dunn) *April* 1157

——— Development of spontaneous tumor in agent-free C3Hb mice. (Heston, Deringer, Dunn, and Levillain) *April* 1139

——— Influence of number of litters on incidence of tumors in mice. (Muhlbock) *June* 1139

——— Tumors in mice containing milk agent, effect of penicillin on. (Bennison) *August* 175

——— Tumor agent, disappearance of, in mice of strains C3H and C. (Andervont) *August* 201

——— Tumor incidence in mice of strain C3H and descendants of foster strain C. (Andervont) *August* 193

Page

Cancer, urease in human stomach with respect to acid secretion in. (Glick) *October* 321
Carbamates, substituted, inhibition of sea-urchin egg cleavage by. (Cornman) *April* 1123
Carcinogenic action (2-nitro, 2-amino, 2-acetylamino, and 2-diacetylaminofluorene) in rat after ingestion and painting. (Morris, Dubnik, and Johnson) *June* 1201
Carcinogenic hydrocarbons in oil emulsions, histogenesis of carcinoma of forestomach of mice fed with. (Stewart and Lorenz) *August* 147
Carcinogens, chemical, effect on growth of normal plant tissue *in vitro*. Cytology of carrot-root tissue (Levine) *April* 1005
Carcinoma, adeno-, of breast, in Wistar rat following gastric instillation of methylcholanthrene. (Shay, Aegerter, Gruenstein, and Komarow) *October* 255
——— Adeno-, of glandular stomach of mice following 20-methylcholanthrene. (Stewart, Hare, Lorenz, and Bennett) *October* 359
——— Cells, gastric aspirate for cytologic study. (Bryant, Craig, and Pollard) *October* 459
——— Of forestomach in mice fed carcinogenic hydrocarbons in oil emulsions; morbid anatomy, histopathology, and histogenesis. (Stewart and Lorenz) *August* 147
——— Gastric, effect of intravenous glucose on gastric lactic acid in. (Shacter, Byron, and Shimkin) *October* 331
——— Metastatic mammary, transplanted subcutaneously into foreign strain mice; lack of progressive growth. (Prehn) *February* 893
——— Tar-induced, biotherapeutic action of *Trypanosoma cruzi*. (Jedeloo, Lignac, Ligtenberg, and van Thiel) *February* 809
Cardiovascular effects produced by stilbamidine in patients with multiple myeloma. (Bierman and Sokolow) *October* 279
Carrot-root tissue, cytology, as affected by chemical carcinogens. (Levine) *April* 1005
Catecholase and catecholase inhibitor activity of human blood serum. (Shacter and Shimkin) *December* 637
Cellophane, influence of perforation design on growth of tissue cells *in vitro*. (Earle, Evans, Edward, and Duchesne) *October* 291
——— Substrate, extension of procedure to growth of *in vitro* cultures of large areas. (Earle, Evans, and Schilling) *February* 943
——— Substrate, perforated, in tissue cultures. (Schilling, Earle, and Evans) *February* 883
Cells at fluid interface of cultures; production of malignancy *in vitro*. (Earle, Schilling, and Shelton) *February* 865
——— At glass interface of cultures; production of malignancy *in vitro*. (Earle, Schilling, and Shelton) *April* 1067
Central nervous system, fluorescein dyes in diagnosis of tumor of, (Hubbard and Moore) *October* 303
Chicken Tumor I (Rous Sarcoma); effects *in vitro* of X radiation on biological activity of agent. (Bryan, Lorenz, and Moloney) *June* 1215
——— Stability of agent in phosphate and borate buffers during incubation. (Bryan, Maver, Moloney) *December* 647
Coproporphyrins, urinary, in patients with neoplastic diseases treated with methyl-bis(β-chloroethyl)amine hydrochloride (HN_2). (Bierman, Strait, and Hrenoff) *August* 93
Cultures *in vitro*, extension of cellophane substrate procedure to growth of, (Earle, Evans, and Schilling) *February* 943
Cytohistologic study of gastric washings. (Richardson) *October* 467
Cytologic study of gastric aspirate for carcinoma cells. (Bryant, Craig, and Pollard) *October* 459

Page

DDT, observations on inbred mice exposed to, (Bennison and Mostofi) *February* 989
Dehydrogenase activity of certain tumors and normal tissues. (Meister) *June* 1263
Detergent, synthetic; formation of tyrosine crystals from leukocytes and tissues by means of, (Ayres) *June* 1239
Diagnosis of malignant disease, fluorescein dyes in, (Hubbard and Moore) *October* 303
p-Dimethylaminoazobenzene containing isotopic carbon in dimethylamino group, preparation of, (Fones and White) *December* 663
Diphosphopyridine nucleotide—cytochrome *c* reductase in normal mouse liver and mouse hepatoma. (Hogeboom and Schneider) *February* 983
Dog, effect of ectopic autologous grafts of androgen-stimulated prostate upon serum "acid" phosphatase of, (Hertz, Westfall, Barrett, and Tullner) *August* 61
——— Inhibition of gastric secretion in, (Blackburn and Code) *October* 337
Eclampsia-like condition in pregnant rats injected with progesterone. (Symeonidis) *December* 711
Endocrine factors, histogenesis and influence of, on gastric neoplasia in mice. (Smith and Strong) *October* 423
Enzyme systems, related, neoplastic transformation as biological fractionation of, (Greenstein, Fodor, and Leuthardt) *October* 271
Esophagoscope, use of, for obtaining biopsies of gastric tumors. (Patterson) *October* 477
Estrogen-induced tumors in guinea pigs, histopathology of, (Nadel) *April* 1043
Eugenol, histologic study of gastric mucous barrier following application of, (Hollander and Goldfisher) *October* 339
Excerpta Medica (Announcement) *December* 804
Fatty acid oxidation, by normal and neoplastic liver. (Baker and Meister) *April* 1191
Fibrosarcoma, transplanted, changes in, associated with ascorbic acid deficiency. (Robertson, Dalton, and Heston) *August* 53
Fluorene, 2-nitro-, 2-amino-, 2-acetylamino-, and 2-diacetylamino-, action in rat after ingestion and painting. (Morris, Dubnik, and Johnson) *June* 1201
Fluorescein dies in diagnosis of malignancy, with special reference to tumors of central nervous system. (Hubbard and Moore) *October* 303
Folic-acid antagonist on transplantable lymphoid leukemia in mice, effect of, (Law, Dunn, Boyle, and Miller) *August* 179
Gastric acidity in adenomatous gastric polyp. (Cromer, Comfort, Butt, and Berkson) *October* 497
——— Aspirate for carcinoma cells; cytologic study. (Bryant, Craig, and Pollard) *October* 459
——— Cancer, attempt to identify precursor groups. (State, Gaviser, Hubbard, and Wangensteen) *October* 443
——— Cancer, etiologic implications of racial incidences. (Steiner) *October* 429
——— Cancer, experimentally induced in insects by nerve severance, (Scharrer) *October* 375
——— Cancer, malignancy of, (Meissner) *October* 533
——— Cancer, metabolic changes in patients with, (Homburger, Young, Potor, Trunnell, Duffy, Peterman, and Rhoads) *October* 489
——— Cancer, photofluorography in detection of, (Roach, Sloan, and Morgan) *October* 455
——— Cancer, problems of Washington State tumor registry regarding, (Chipps) *October* 439
——— Cancer, silent interval in (Friesen) *October* 545
——— Carcinoma, acid-phosphatase in gastric content of patients with, (Changus and Dunlap) *October* 481

Page

Gastric Lesions in rat as result of feeding tributyrin. (Salmon and Copeland) *October*) ... 361
—— Mucin, human, inhibition of gastric secretion in dog by, (Blackburn and Code) *October* ... 337
——— Mucosa, lesion in strain NHO mice, (Kaplan) *October* ... 407
——— Mucous barrier, histologic study of destruction and regeneration of, following eugenol, (Hollander and Goldfischer) *October* ... 339
———Neoplasia in mice; histogenesis and influence of endocrine factors, (Smith and Strong) *October* ... 423
——— Neoplasms, mucous cells in production of (Howes) *October* ... 377
——— Secretion, inhibition of, in dog by human gastic juice and gastric mucin, (Blackburn and Code) *October* ... 337
———Tumor, esophagoscope for obtaining biopsy, (Patterson) *October* ... 477
——— Washings, cytohistologic study, (Richardson) *October* ... 467
Gastritis, chronic, in cancer-bearing stomachs; topography, (Hebbel) *October* ... 505
——— Chronic atrophic, identification of different varieties, (Cox) *October* ... 523
Gene, hairless, relationship between and susceptibility to induced pulmonary tumors in mice, (Heston and Deringer) *August* ... 119
Glass cloth, substrate for tissue culture, (Warner, Hanawalt, and Bischoff) *August* ... 67
Glucose, intravenous, effect on gastric lactic acid in gastric carcinoma, (Shacter, Byron, and Shimkin) *October* ... 331
Glucuronidase activity in gastrointestinal neoplasms, (Fishman and Bigelow) *April* ... 1115
——— Liver, in inbred mice, (Morrow, Greenspan, and Carroll) *April* ... 1199
——— Liver, in inbred mouse strains, (Morrow) *December* ... 657
Gold implants, apparatus for measuring, (Rose and Swain) *December* ... 605
Grafts, ectopic autologous, of androgen-stimulated prostate; effect on serum "acid" phosphatase of dog, (Hertz, Westfall, Barrett, and Tullner) *August* ... 61
Growth of transplantable tumor in hybrid mice, maternal influence on rate of, (Barrett and Morgan) *August* ... 81
Guinea pig, action of 9,10-dimethyl-1,2-benzanthracene on skin and subcutaneous tissue, (Berenblum) *August* ... 167
——— Estrogen-induced tumors in, (Nadel) *April* ... 1043
Hairless gene relation between . . . and susceptibility to induced pulmonary tumors in mice. (Heston and Deringer) *August* ... 119
Hamster, relation of duration of transplanted sarcoma to metastasis in, (Crabb) *December* ... 581
——— Tumor-bearing, hematopoiesis in spleen of, (Kelsall) *December* ... 625
Hemangioendothelioma and sarcoma in mice, induction with *o*-aminoazotoluene, (Andervont) *February* ... 927
Hematopoiesis in spleen of tumor-bearing hamsters. (Kelsall) *December* ... 625
Hepatoma 31, dietary influence of protein, riboflavin, and biotin on growth of malignant and nonmalignant tissues in rat bearing, (Voegtlin and Thompson) *August* ... 29
Hepatoma, lactonase and C-acylase activity of, (Meister) *August* ... 75
Histamine content of experimental tumors. (Rosenthal) *August* ... 89
Histochemical Society, Business meeting, *June* ... 1335
——— Index of authors ... 1339
——— Scientific Proceedings ... 1341
Hyaluronidase in human and animal tumors and studies on serum inhibitor in human cancer; mucolytic enzyme studies. (Kiriluk, Kremen, and Glick) *February* ... 993
Hydrocarbons, carcinogenic; action of 9,10-dimethyl-1,2-benzanthracene on skin and subcutaneous tissue of mouse, rabbit, rat, and guinea pig. (Berenblum) *August* ... 167
——— Preparation of emulsions and suspensions containing, (Lorenz) *October* ... 355

Page

Inhibition of mammary-gland development and mammary-tumor formation in female C3H mice following thiouracil. (Dubnik, Morris, and Dalton) *February* 815
Insects, gastric cancer induced in, by nerve severance. (Scharrer) *October* 375
Intestine, study of morphology and glucuronidase activity in neoplasms of, (Fishman and Bigelow) *April* 1115
Iodoacetate index, antiproteolytic reaction, and least coagulable protein reactions in relation to malignant neoplasia. (Cliffton) *December* 719
p-Iodobenzene diazonium chloride, radioactive, . . . experimental and clinical study. Chemical alteration of polysaccharide from *Serratia marcescens*. (Seligman and Sack) *August* 105
Irradiation, local, in induction of lymphoid tumors in mice; preliminary studies of effectiveness. (Kaplan) *October* 267
Lactic acid in gastric carcinoma; effect of intravenous glucose. (Shacter, Byron, and Shimkin) *October* 331
Lactonase and C-acylase activity of hepatoma. (Meister) *August* 75
Lesion of gastric mucosa in strain NHO mice. (Kaplan) *October* 407
Leukemia, lymphoid, transplantable in mice; effect of folic-acid antagonist. (Law, Dunn, Boyle, and Miller) *August* 179
Leukocytes and normal and pathological tissue; formation of tyrosine crystals from, by synthetic detergent. (Ayres) *June* 1239
Liver, studies on fatty oxidation by normal and neoplastic, (Baker and Meister) *April* 1191
——— Glucuronidase activity of inbred mouse strains. (Morrow) *December* 657
——— Glucuronidase activity in inbred mice (Morrow, Greenspan, and Carroll) *April* 1199
Lung, induction of tumors in strain A mice with methyl-bis (β-chloroethyl) amine hydrochloride. (Heston) *August* 125
——— Susceptibility of induced pulmonary tumors in hairless gene (Heston and Deringer) *August* 119
Lymphoma, effect of alpha-peltatin, beta-peltatin, and podophyllotoxin on, (Greenspan, Leiter, and Shear) *June* 1295
Malignancy *in vitro;* description of cells at glass interface of cultures. (Earle, Schilling, and Shelton) *April* 1067
——— Results from reinjection of cell strains into C3H mice. (Earle, Shelton, and Schilling) *April* 1105
Mammary-gland development and tumor formation in C3H mice following thiouracil, inhibition of, (Dubnik, Morris, and Dalton) *February* 815
Maternal influence on growth rate of transplantable tumor in hybrid mice. (Barrett and Morgan) *August* 81
Melanin production, cutaneous, in mice following 5,9,10-trimethyl-1,2-benzanthracene. (Burgoyne, Heston, and Hartwell) *December* 665
Melanoblasts in stroma of parathyroid gland of strain C58 mice. (Dunn) *December* 725
Metabolic changes in patients with gastric cancer. (Homburger, Young, Potor, Trunnell, Duffy, Peterman, and Rhoads) *October* 489
Metabolism of stomach. (Davenport) *October* 315
Methyl-bis (β-chloroethyl) amine hydrochloride, induction of pulmonary tumors in strain A mice with, (Heston) *August* 125
——— (β-chloroethyl) amine hydrochloride (HN_2), urinary coproporphyrins in patients with neoplastic diseases treated with, (Bierman, Strait, and Hrenoff) *August* 93
Methylcholanthrene, adenocarcinoma of breast in rat following gastric instillation of, (Shay, Aegerter, Gruenstein, and Komarow) *October* 255
——— Adenocarcinoma of glandular stomach of mice following injection. (Stewart, Hare, Lorenz, and Bennett) *October* 359

Page

Methylcholanthrene, Analysis of dose response data; induced pulmonary tumors in mice (Shimkin and McClelland) *December* 597
——— Response of mammary-tumor-agent-free strain dba female mice to percutaneous application of (Andervont and Dunn) *February* 895
Milk agent, effect of penicillin on mammary tumors in mice containing, (Bennison) *August* 175
Mouse, action of 9,10-dimethyl-1,2-benzanthracene on skin and subcutaneous tissue. (Berenblum) *August* 167
——— Adenocarcinoma of glandular stomach following 20-methylcholanthrene. (Stewart, Hare, Lorenz, and Bennett) *October* 359
——— Biotherapeutic action of *Trypanosoma cruzi* on tar carcinoma in, (Jedeloo, Lignac, Ligtenberg, and van Thiel) *February* 809
——— C3H and descendants of foster strain C; mammary tumor incidence. (Andervont) *August* 193
——— Containing milk agent; effect of penicillin in mammary tumors. (Bennison) *August* 175
——— Cutaneous melanin production following 5,9,10-trimethyl-1, 2-benzanthracene. (Burgoyne, Heston, and Hartwell) *December* 665
——— Effect of folic-acid antagonist on transplantable lymphoid leukemia. (Law, Dunn, Boyle, and Miller) *August* 179
——— Effectiveness of local irradiation in induction of lymphoid tumor. (Kaplan) *October* 267
——— Effects of podophyllin on skin. (King and Cauldwell) *August* 131
——— Effects of podophyllin on skin of; a study of epidermal fibrils. (King) *December* 689
——— Exposed to DDT; observations. (Bennison and Mostofi) *February* 989
——— Fed carcinogenic hydrocarbons in oil emulsion; histogenesis of carcinoma of forestomach. (Stewart and Lorenz) *August* 147
——— Gastric neoplasia; histogenesis and influence of endocrine factors. (Smith and Strong) *October* 423
——— Hairless gene; susceptibility to induced pulmonary tumors. (Heston and Deringer) *August* 119
——— High-cancer-strain; mammary tumor-agent in sperm. (Muhlbock) *February* 861
——— Hybrid; maternal influence on growth rate of transplantable tumor. (Barrett and Morgan) *August* 81
——— Inbred strains; liver glucuronidase activity. (Morrow) *December* 657
——— Inbred; studies of liver glucuronidase activity. (Morrow, Greenspan, and Carroll) *April* 1199
——— Induced pulmonary tumors with methylcholanthrene; analysis of dose response data. (Shimkin and McClelland) *December* 597
——— Induction of hemangioendothelioma and sarcoma with *o*-aminoazotoluene. (Andervont) *February* 927
——— Influence of number of litters on incidence of mammary tumors. (Muhlbock) *June* 1259
——— Inhibition of mammary-gland development and mammary-tumor-formation in female C3H following thiouracil. (Dubnik, Morris, and Dalton) *February* 815
——— Lack of growth of metastatic mammary carcinoma transplanted into foreign strain. (Prehn) *February* 893
——— Mammary-tumor-agent-free strain dba female; response to methylcholanthrene. (Andervont and Dunn) *February* 895
——— Strain I, spontaneous lesion of stomach in. (Andervont) *October* 405
——— Strain A, induction of pulmonary tumors with methyl-bis (β-chloroethyl)amine hydrochloride. (Heston) *August* 125
——— Strain C, attempt to detect mammary tumor by x-radiation. (Andervont and Dunn) *April* 1157

Page

Mouse, Strain C3H and C, disappearance of mammary-tumor-agent. (Andervont) *August* 201
——— Strain C3H, results from reinjection of *in vitro* cell strains into. (Earle, Shelton, and Schilling) *April* 1105
——— Strain C58, melanoblasts in stroma of parathyroid gland. (Dunn) *December* 725
——— Strain NHO, lesion of gastric mucosa. (Kaplan) *October* 407
——— Transparent-chamber technique as adapted to, (Algire and Legallais) *October* 225
Mucous barrier, gastric, destruction and regeneration of, (Hollander and Goldfischer) *October* 339
Mucous cells in production of gastic neoplasms. (Howes) *October* 377
Myeloma, multiple, cardiovascular effects produced by stilbamidine in patients with, (Bierman and Sokolow) *October* 279
Neoplasms, gastrointestinal; study of morphology and glucuronidase activity. (Fishman and Bigelow) *April* 115
Neoplastic transformation as biological fractionation of related enzyme systems. (Gruenstein, Fodor, and Leuthardt) *October* 271
Nerve severance, gastric cancer induced in insects by, (Scharrer) *October* 375
Nucleic acids and adenosinetriphosphatase in normal mouse liver and mouse hepatoma; intracellular distribution of enzymes. (Schneider, Hogeboom, and Ross) *February* 977
Paramecium, variations in life expectancy. (Spencer and Melroy) *August* 1
Parathyroid gland of strain C58 mice, melanoblasts in stroma of, (Dunn) *December* 725
Penicillin, effect on mammary tumors in mice containing milk agent. (Bennison) *August* 175
Phosphatase, "acid", of dog, effect of ectopic autologous grafts of androgen-stimulated prostate upon, (Hertz, Westfall, Barrett, and Tullner) *August* 61
Phosphate buffer, comparative stability of agent of Chicken Tumor I. (Bryan, Maver, Moloney, Thomas, and Sells) *December* 647
Photofluorography in detection of gastic cancer. (Roach, Sloan, and Morgan) *October* 455
Podophyllin, damage induced in Sarcoma 37 with, (Leiter, Downing, Hartwell, and Shear) *June* 1273
——— Effects on mouse skin; a study of epidermal fibrils. (King) *December* 689
——— Effects on mouse skin; functional aspects. (King and Cauldwell) *August* 131
Podophyllotoxin, damage induced in Sarcoma 37 with, (Leiter, Downing, Hartwell, and Shear) *June* 1273
——— Effect on lymphomas and other transplanted tumors. (Greenspan, Leiter, and Shear) *June* 1295
Polyp, gastric, adenomatous, gastric acidity in, (Cromer, Comfort, Butt, and Berkson) *October* 497
Polysaccharide, chemical alteration from *Serratia marcescens*. (Seligman and Sack) *August* 105
Proceedings of fourth conference on gastic cancer. *October* 297
——— First meeting of Histochemical Society. *June* 1335
Progesterone, eclampsia-like condition in pregnant rats injected with, (Symeonidis) *December* 711
Prostate, androgen-stimulated, effect of ectopic autologous grafts upon serum "acid" phosphatase of dog. (Hertz, Westfall, Barrett, and Tullner) *August* 61
Protein, dietary, influence in growth of malignant and nonmalignant tissues in rat bearing Hepatoma 31. (Voegtlin and Thompson) *August* 29
Quercetin, damage induced in Sarcoma 37 with, (Leiter, Downing, Hartwell, and Shear) *June* 1273

Page

Rabbit, action of 9,10-dimethyl-1,2-benzanthracene on skin and subcutaneous tissue. (Berenblum) *August* 167
Racial incidences of gastric cancer; etiologic implications. (Steiner) *October* 429
Radiation injury to amphibian cell nuclei. (Duryee) *December* 735
Radium, remote-controlled; emanation plant, and automatic measuring apparatus for gold implants. (Rose and Swain) *December* 605
Rat, action of 9,10-dimethyl-1,2-benzanthracene on skin and subcutaneous tissue. (Berenblum) *August* 167
——— Carcinogenic action of 2-nitro-, 2-amino-, 2-acetylamino, and 2-diacetylaminofluorene. (Morris, Dubnik, and Johnson) *June* 1201
——— Carcinoma, adeno-, in breast following gastric methylcholanthrene. (Shay, Aegerter, Gruenstein, and Komarow) *October* 255
——— Gastric lesion, as result of feeding tributyrin. (Salmon and Copeland) *October* 361
——— Influence of dietary protein, riboflavin, and biotin in growth of malignant and nonmalignant tissues in rats bearing Hepatoma 31. (Voegtlin and Thompson) *August* 29
——— Pregnant, injected with progesterone; eclampsia-like condition. (Symeonidis) *December* 711
Registry, tumor (Washington State); problems regarding gastric cancer and cancer research. (Chipps) *October* 439
Riboflavin, dietary, influence in growth of malignant and nonmalignant tissues in rat bearing Hepatoma 31. (Voegtlin and Thompson) *August* 29
Rous Sarcoma, See Chicken Tumor I.
Sarcoma, induction with *o*-aminoazotoluene. (Andervont) *February* 927
——— 37, damage induced with podophyllin, podophyllotoxin, alpha-peltatin, beta-peltatin, and quercetin. (Leiter, Downing, Hartwell, and Shear) *June* 1273
——— Fibro-, transplanted, changes in, associated with ascorbic acid deficiency. (Robertson, Dalton, and Heston) *August* 53
——— Sarcoma, transplanted, relation of duration to metastasis in hamster. (Crabb) *December* 581
Sea-urchin egg cleavage, inhibition of, by substituted carbamates. (Cornman) *April* 1123
Serratia marcescens, chemical alterations of polysaccharide from; reaction with radioactive *p*-iodobenzene diazonium chloride and use of product in experimental and clinical study. (Seligman and Sack) *August* 105
Skin, action of 9,10-dimethyl-1,2-benzanthracene on, in mouse, rabbit, rat, and guinea pig. (Berenblum) *August* 167
Sperm of high-cancer-strain mice, mammary tumor-agent in, (Muhlbock) *February* 861
Spleen, hematopoiesis of, in tumor-bearing hamsters. (Kelsall) *December* 625
Spontaneous lesion of stomach in strain I mice. (Andervont) *October* 405
Stomach, histogenesis of carcinoma in mice fed carcinogenic hydrocarbons. (Stewart and Lorenz) *August* 147
——— Glandular, of mice; adenocarcinoma following 20-methylcholanthrene. (Stewart, Hare, Lorenz, and Bennett) *October* 359
——— Human, urease in, with respect to acid secretion in ulcer and cancer. (Glick) *October* 321
——— Metabolism of, (Davenport) *October* 315
——— Spontaneous lesion in strain I mice. (Andervont) *October* 405
——— Study of morphology and glucuronidase activity in neoplasms of, (Fishman and Bigelow) *April* 1115
——— Topography of chronic gastritis in cancer-bearing, (Hebbel) *October* 505
Stilbamidine, cardiovascular effects produced in patients with multiple myeloma. (Bierman and Sokolow) *October* 279
Substrate for tissue culture, glass cloth as, (Warner, Hanawalt, and Bischoff) *August* 67

Page

Succinoxidase and cytochrome oxidase activities in normal mouse liver and in mouse hepatoma; intracellular distribution of enzymes. (Schneider and Hogeboom) *February* 969

Thiouracil, inhibition of mammary-gland development and mammary-gland formation in female C3H mice following ingestion of, (Dubnik, Morris, and Dalton) *February* 815

Tissue-cell growth under perforated cellophane *in vitro;* influence of perforation design. (Earle, Evans, Edward, and Duchesne) *October* 291

Tissue-culture, glass cloth as substrate. (Warner, Hanawalt, and Bischoff) *August* 67

——— Perforated cellophane substrate. (Schilling, Earle, and Evans) *February* 883

Tissue, differential growth of malignant and nonmalignant in rats bearing Hepatoma 31; influence of dietary protein, riboflavin, and biotin. (Voegtlin and Thompson) *August* 29

——— Normal, dehydrogenase activity of, (Meister) *June* 1263

——— Subcutaneous, action of 9,10-dimethyl-1,2-benzanthracene on, in mouse, rabbit, rat, and guinea pig. (Berenblum) *August* 167

Transparent-chamber technique as adapted to mouse. (Algire and Legallais) *October* 225

Tributyrin, gastric lesions in rat as result of feeding. (Salmon and Copeland) *October* 361

Trypanosoma cruzi, action of, on tar carcinoma in mice. (Jedeloo, Lignac, Ligtenberg, and van Thiel) *February* 809

Tumor, central nervous system, fluorescein dyes in diagnosis of, (Hubbard and Moore) *October* 303

——— Dehydrogenase activity of, (Meister) *June* 1263

——— Estrogen-induced in guinea pig, histopathology of, (Nadel) *April* 1043

——— Experimental, histamine content of, (Rosenthal) *August* 89

——— Gastric, esophagoscope for obtaining biopsy (Patterson) *October* 477

——— Gastric, histogenesis and influence of endocrine factors. (Smith and Strong) *October* 423

——— Gastric, mucous cells in production of, (Howes) *October* 377

——— Incidence in breast of mice of strain C3H and in descendants of foster strain C. (Andervont) *August* 193

——— Lymphoid, in mice, effect of local irradiation in induction of, (Kaplan) *October* 267

——— Malignant, iodoacetate index and least coagulable protein reactions in relation to, (Cliffton) *December* 719

——— Mammary, in mice containing milk agent; effect of penicillin. (Bennison) *August* 175

——— Mammary, in mice; influence of number of litters on incidence. (Muhlbock) *June* 1259

——— Pulmonary in strain A mice, induced with methyl-bis(β-chloroethyl)-amine hydrochloride. (Heston) *August* 125

——— Pulmonary, induced with methylcholanthrene; analysis of dose response data. (Shimkin and McClelland) *December* 597

——— Spontaneous, of mammary gland in agent-free C3Hb mice; factors in development. (Heston, Deringer, Dunn, and Levillain) *April* 1139

——— Susceptibility to induced pulmonary, in mice of hairless gene. (Heston and Deringer) *August* 119

——— Transplantable in hybrid mice; maternal influence on growth rate. (Barrett and Morgan) *August* 81

——— Transplantable lymphoid leukemia in mice; effect of folic-acid antagonist. (Law, Dunn, Boyle, and Miller) *August* 179

——— Transplanted; effect of alpha-peltatin, beta-peltatin, and podophyllotoxin. (Greenspan, Leiter, and Shear) *June* 1295

Page

Tumor, Transplanted; effect of vascular occlusion. (Youngner and Algire) *December* ---- 565
Tumor-agent, mammary; attempt to detect in strain C mice by X-radiation. (Andervont and Dunn) *April* ---- 1157
——— Mammary, in sperm of high-cancer-strain mice. (Muhlbock) *February* ---- 861
——— Mammary, disappearance in mice of Strains C3H and C. (Andervont) *August* ---- 201
Tumor-formation, mammary, in C3H mice following thiouracil; inhibition of, (Dubnik, Morris, and Dalton) *February* ---- 815
Tyrosine crystals, formation from leukocytes and normal and pathological tissue by synthetic detergent. (Ayres) *June* ---- 1239
Ulcer, urease in human stomach with respect to acid secretion in, (Glick) *October* ---- 321
Ultrasonic vibration on formed elements of blood from normal and leukemic subjects, effect of, (Morrow, Bierman, and Jenkins) *February* ---- 843
Unicellular species, bacterial, principle involved in survival. (Melroy and Spencer) *August* ---- 11
Urease in human stomach with respect to acid secretion in ulcer and cancer. (Glick) *October* ---- 321
Urinary coproporphyrins in patients with neoplastic diseases treated with methyl-bis(β-chloroethyl)amine hydrochloride (HN_2). (Bierman, Strait, and Hrenoff) *August* ---- 93
Vascular occlusion; effect on transplanted tumors. (Youngner and Algire) *December* ---- 565
Washington State Tumor Registry; problems regarding gastric cancer and cancer research. (Chipps) *October* ---- 439
X-radiation, attempt to detect mammary tumor-agent in strain C mice. (Andervont and Dunn) *April* ---- 1157
X-radiation, study of effects *in vitro* on biological activity of agent of Chicken Tumor I (Rous Sarcoma). (Bryan, Lorenz, and Moloney) *June* ---- 1215

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CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 10 NO. 6 JUNE 1950

Page

Studies of the carcinogenic action in the rat of 2-nitro-, 2-amino-, 2-acetylamino-, and 2-diacetylaminofluorene after ingestion and after painting. *Harold P. Morris, Celia S. Dubnik, and James M. Johnson* 1201

Studies on the effects *in vitro* of roentgen radiation on the biological activity of the agent of chicken tumor I (Rous Sarcoma). *W. Ray Bryan, Egon Lorenz, and John B. Mulcahy* 1215

Formation of tyrosine crystals from leukocytes and various normal and pathological tissues by means of a synthetic detergent. *William W. Ayres* 1239

Note on the influence of the number of litters upon the incidence of mammary tumors in mice. *O. Muhlbock* 1259

Lactic dehydrogenase activity of certain tumors and normal tissues. *Alton Meister* 1263

Damage induced in Sarcoma 37 with podophyllin, podophyllotoxin, alpha-peltatin, beta-peltatin, and quercetin. *J. Leiter, V. Downing, J. L. Hartwell, and M. J. Shear* 1273

Effect of alpha-peltatin, beta-peltatin, and podophyllotoxin on lymphomas and other transplanted tumors. *Ezra M. Greenspan, J. Leiter, and M. J. Shear* 1295

Histochemical Society, Business Meeting 1334

Index of authors 1339

Scientific Proceedings 1341

Index for Volume 10 1383

JOURNAL OF THE NATIONAL CANCER INSTITUTE

[INDEX]

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